(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 June 2001 (28.06.2001)

PCT

(10) International Publication Number
WO 01/46455 A2

(51) International Patent Classification[7]: C12Q

(21) International Application Number: PCT/US00/34663

(22) International Filing Date:
21 December 2000 (21.12.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/172,991 21 December 1999 (21.12.1999) US

(71) Applicant *(for all designated States except US)*: **YALE UNIVERSITY** [US/US]; 451 College Street, New Haven, CT 06520 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ALTIERIA, Dario, C.** [IT/US]; Boyer Center for Molecular Medicine, Rm 436B, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06536 (US). **SESSA, William, A.** [US/US]; Boyer Center for Molecular Medicine, Rm 436D, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06536 (US).

(74) Agent: **TENG, Sally, P.**; Morgan, Lewis & Bockius LLP, 1800 M Street, N.W., Washington, DC 20036-5869 (US).

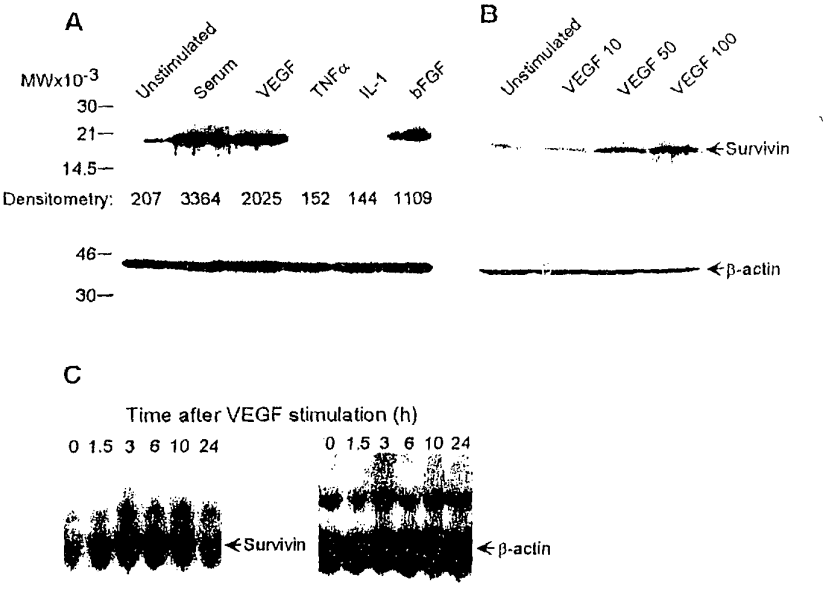
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

[Continued on next page]

(54) Title: SURVIVIN PROMOTION OF ANGIOGENESIS



(57) Abstract: The present invention discloses methods for promoting angiogenesis using agents that increase the activity function and/or expression of survivin. The present invention also discloses methods for inhibiting angiogenesis using agents that inhibit activity, the function and/or expression of survivin.

WO 01/46455 A2

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

SURVIVIN PROMOTION OF ANGIOGENESIS

INVENTORS: Dario C. Altieri and William C. Sessa

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/172,991, filed December 21, 1999, which is herein incorporated by reference in its entirety. This application is related to U.S. application 08/975,080, filed November 20, 1997, which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

The invention relates generally to the modulation of survivin to induce or inhibit angiogenesis.

BACKGROUND OF THE INVENTION

The genetic control of cell death/viability (apoptosis) preserves tissue and organ homeostasis by eliminating senescent or damaged cells (Vaux *et al.*, 1999). This process involves different gene families of inhibitors and stimulators of cell death, and culminates with activation of intracellular cysteine proteases known as caspases (Salvesen *et al.*, 1997). Aberrations of the apoptosis moieties are known to contribute to human diseases, including cancer (Thompson, 1995) and vascular disorders (Rudin *et al.*, 1997). Specifically, aberrantly increased cell death has been shown to influence atherosclerotic plaque instability (Bjorkerud *et al.*, 1996), congestive heart failure (Olivetti *et al.*, 1997), coronary disease (Olivetti *et al.*, 1996), and ischemic neuronal loss (Chen *et al.*, 1998).

The endothelium is one of the most critical sites for the control of apoptosis in vascular injury and vascular remodeling (Karsan *et al.*, 1996). In inflammation, a heterogeneous group of "protective"genes activated by nuclear factor κB, opposes cell death and proinflammatory changes in endothelial cells (EC) induced by cytokines, *i.e.* tumor necrosis factor α (TNFα) (Bach *et al.*, 1997). Inhibition of apoptosis may also be

obligatorily required during vascular remodeling and new blood vessel formation (Risau, 1997). In this context, EC specific mitogens, including vascular endothelial cell growth factor (VEGF) or basic fibroblast growth factor (bFGF), transduce survival signals critically maintaining EC viability, *in vivo* (Benjamin *et al.*, 1997; Alon *et al.*, 1995; Yuan *et al.*, 1996). However, the downstream effector genes coupling mitogen-dependent survival to the anti-apoptotic machinery in EC have not been completely elucidated.

It has been shown that angiopoietin-1 (Ang-1) is an endothelium-specific ligand essential for embryonic vascular stabilization, branching morphogenesis, and post-natal angiogenesis. During angiogenesis, EC receive cues from growth factors to initiate mitosis, migration and organization of endothelial cells into primitive angiotubes and patent vascular networks (Risau 1997; Hanahan 1997). These processes critically depend on preservation of endothelial cell viability. Disruption of endothelial cell-matrix contacts or interference with extracellular survival signals is sufficient to initiate caspase-dependent apoptosis in endothelium, culminating with rapid involution of vascular structures (Brooks *et al.*, 1994; O'Reilly *et al.*, 1996). Unlike most angiogenic regulators, including fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF), Ang-1 does not stimulate endothelial cell growth, but rather promotes stabilization of vascular networks and branching morphogenesis, *in vivo* and *in vitro* (Davis *et al.*, 1996; Koblizek *et al.*, 1998; Papapetropoulos *et al.*, 1999; Witzenbichler *et al.*, 1998). Little is known about the signaling requirements of these responses, and it is unclear if the role of Ang-1 in angiogenesis includes protection of EC from apoptosis (Papapetropoulos *et al.*, 1999; Kontos *et al.*, 1998).

Numerous inhibitors of apoptosis (IAP) characterized with anti-apoptotic functions have been identified. These molecules are highly conserved evolutionarily; they share a similar architecture organized in two or three approximately 70 amino acid amino terminus Cys/His baculovirus IAP repeats (BIR) and by a carboxy terminus zinc-binding domain, designated RING finger (Duckett *et al.*, 1996; Hay *et al.*, 1995; Liston *et al.*, 1996; Rothe, M. *et al.*, 1995; Roy *et al.*, 1995). Recombinant expression of IAP

proteins blocks apoptosis induced by various stimuli *in vitro* (Duckett *et al.*, 1996; Liston *et al.*, 1996), and promotes abnormally prolonged cell survival in the developmentally-regulated model of the Drosophila eye, *in vivo* (Hay *et al.*, 1995).

Survivin has recently been identified as a novel member of the IAP family. Survivin is a 16.5 kDa cytoplasmic protein containing a single partially conserved BIR domain, and a highly charged carboxyl-terminus coiled-coil region instead of a RING finger, which inhibits apoptosis induced by growth factor (IL-3) withdrawal when transferred in B cell precursors (Ambrosini *et al.*, 1997). Based on overall sequence conservation, the absence of a carboxy terminus RING finger and the presence of a single, partially conserved, BIR domain, survivin is the most distantly related member of the IAP family, sharing the highest degree of similarity with NAIP (Roy *et al.*, 1995). Additionally, unlike other IAP proteins, survivin is undetectable in adult tissues, but becomes prominently expressed in all the most common human cancers of lung, colon, breast, pancreas, and prostate, and in ~50% of high-grade non-Hodgkin's lymphomas, *in vivo*. Moreover, survivin does not bind caspases in a cell-free system (Roy *et al.*, 1997). Although survivin has been characterized as a cell cycle regulated apoptosis inhibitor, the role of survivin on EC viability and angiogenesis has not been previously discovered.

Angiogenesis, *i.e.* the formation of new blood vessels from existing ones (Risau, W., 1997) is an indispensable process for organ and tissue development, and genetic dysregulation of these mechanisms has been associated with embryonic lethality (Hanahan, D. 1997). In the adult organism, angiogenesis provides for beneficial compensatory mechanisms that increase blood supply in response to hypoxia or during tissue repair and/or remodeling (Carmeliet, P., 2000). The same process, however, may have disastrous consequences in cancer, where increased vascularization due to *de novo* vessel formation contributes to tumor progression and metastatic dissemination (Hanahan *et al.*, 1996). Targeted inhibition of angiogenesis by either disrupting cell-cell (Stromblad *et al.*, 1996) and cell-matrix interactions (Brooks *et al.*, 1998) or by interfering with receptor-initiated intracellular signals (Lin *et al.*, 1998; Claeson-Welsh *et al.*, 1998), results in rapid involution of newly formed blood vessels *in vitro* and *in vivo*,

and the appearance of classical morphologic features of apoptosis in the targeted endothelium (Alon *et al.*, 1995; Yuan *et al.*, 1996; Lin *et al.*, 1998). Accordingly, the continuous suppression of EC apoptosis (Bach *et al.*, 1997) may constitute one of the critical requirements of angiogenesis, consistent with the up regulation of protective genes of the bcl-2 (Gerber *et al.*, 1998a; Nor *et al.*, 1999) or IAP (O'Connor *et al.*, 2000a; Tran *et al.*, 1999; Papapetropoulos *et al.*, 2000) gene families in endothelium stimulated by VEGF or Ang-1.

SUMMARY OF THE INVENTION

The present invention is based on the discovery that angiogenesis stimulation strongly induces survivin expression in endothelium during vascular remodeling and angiogenesis, *in vitro* and *in vivo*. Survivin expression and function affect endothelial cell viability during the proliferative and the stabilizing phase of angiogenesis. Therapeutic manipulation of survivin expression/function in endothelium may influence compensatory or pathologic (tumor) angiogenesis.

The present invention is also based on the discovery that Ang-1 prevents endothelial cell apoptosis by activating a critical survival messenger, Akt, and by up-regulating survivin. The activation of anti-apoptotic pathways mediated by Akt and survivin in endothelial cells may contribute to Ang-1 stabilization of vascular structures during angiogenesis, *in vivo*. Accordingly, targeted manipulation of Ang-1/Akt/Survivin may be exploited to improve endothelial cell viability and favor therapeutic angiogenesis, *in vivo*.

Based on these observations, the present invention provides methods of promoting or inhibiting angiogenesis and methods of treating conditions by inducing compensatory angiogenesis. In one embodiment, the disclosed methods are useful for treating ischemic diseases caused by myocardial infarction, peripheral vascular occlusion, brain ischemia, or stroke. In another embodiment, the disclosed methods of inhibiting angiogenesis are useful for treating vasculoproliferative disease such as cancer, restenosis, vascular bypass graft occlusion, or transplant coronary vasculopathy.

The disclosed methods comprise providing to a cell or tissue an apoptosis modulating agent, wherein the agent is selected from the group consisting of a survivin polypeptide, a survivin transgene, a survivin antisense molecule, a survivin peptidomimetic, or an agent that modulates the expression or activity of survivin in a cell or tissue, such as Ang-1 or Akt. In one embodiment, the agent is provided in an implant. The implant maybe a stent and the implant maybe coated or impregnated with a survivin transgene that is operatively linked to an expression control element in a vector. In another embodiment the survivin transgene or antisense molecule is contained within a transfection facilitating composition, such as a transfection facilitating lipid or a transfection facilitating particle.

In one embodiment, the present invention includes a method of inhibiting angiogenesis comprising administering an agent that inhibits survivin. Examples of agents that inhibit survivin include, but are not limited to, survivin antibodies, survivin antisense molecules, inhibitors of Akt phosphorylation, and other inhibitors of survivin function or expression. In another embodiment, the method inhibits the angiogenesis of tumors or inhibits the metastasis of cancerous cells and the agent is a survivin antisense molecule. Preferably, the method inhibits VEGF induced functions such as ceramide or TNFα induced apoptosis and capillary formation and maintenance. Most preferably, the method inhibits VEGF induced capillary formation and maintenance.

As shown in the Examples below, survivin is involved not only in the proliferative phase of angiogenesis but also the remodeling and stabilizing phase of angiogenesis. Survivin antisense molecule is sufficient to induce endothelial cell apoptosis and regression of the capillary-like structures.

In one aspect, the present invention contemplates the use of inhibitors of survivin expression and function as agents that inhibits angiogenesis, such as an antagonist. In another aspect, the present invention contemplates the use of survivin and inducers of survivin expression and function as agents that promotes angiogenesis, such as an agonist.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-C. Modulation of survivin expression in EC.

A. Quiescent EC were incubated with medium or serum (10% FCS), VEGF (100 ng/ml), bFGF (5 ng/ml), TNFα(10 ng/ml) or IL-1 (2 ng/ml) for 16 h at 37^{0}C. Cells were harvested, SDS-extracted and analyzed for expression of survivin or β-actin, by immunoblotting. **B.** Control or EC were stimulated with the indicated increasing concentrations of VEGF for 16 h at 37°C and analyzed for expression of survivin or β-actin by immunoblotting. **C.** Total RNA was extracted from EC stimulated with 100 ng/ml VEGF at the indicated time intervals, separated on agarose-formaldehyde denaturing gels and hybridized with probes to survivin or control β-actin.

Figures 2A-D. Expression of survivin in three-dimensional EC culture.

EC were grown in three-dimensional fibronectin-collagen gels, paraffin-embedded and analyzed for survivin expression by immunohistochemistry. **A.** Survivin expression in control, two-dimensional EC culture. **B.** Survivin expression in three-dimensional EC culture. **C.** Control staining of three-dimensional EC culture with preimmune antibody. **D.** Two (2-D)- or three (3-D)-dimensional EC cultures were harvested, homogenized in a tissue grinder, and analyzed for survivin expression by immunoblotting.

Figures 3A-F. Expression of survivin in proliferating and non-proliferating skin capillaries.

Five-μm sections of formalin-fixed, paraffin-embedded skin biopsies containing granulation tissue and normal skin were analyzed for survivin expression by immunohistochemistry after antigen retrieval by pressure-cooking. **A.** Strong cytoplasmic expression of survivin in EC of dermal capillaries in granulation tissue. *Inset*, detail representation of dermal capillaries stained for survivin expression. **C.** Expression of survivin in endothelium of large vessel in granulation tissue at the

dermis/hypodermis junction. **B, D.** Control staining for panels **A** and **C**, respectively, in the absence of primary antibody. **E.** Expression of survivin in non-proliferating capillaries of non-inflamed normal skin. **F.** Control incubation for panel E, in the presence of preimmune antibody. Magnification x200 (**A, E, F**) and x400 (**B, C, D**).

Figures 4A-B. Anti-apoptotic function of survivin in EC.

A. Sub-confluent bovine aortic EC were transfected with GFP-vector or GFP-survivin by lipofectin, cultivated for 35 h at 37^{0}C and treated with 5 ng/ml TNFα/5 μg/ml cycloheximide for additional 8-h at 37^{0}C. GFP-expressing cells were analyzed for DNA content by propidium iodide staining and flow cytometry. The percentage of cells with hypodiploid DNA content (sub-G1-fraction) is indicated in parenthesis for each condition tested. **B.** Untreated or EC transfected with GFP-vector or GFP-survivin were incubated with the indicated concentrations of TNFα/10 μg/ml cycloheximide, harvested, and analyzed for caspase-3 activity by hydrolysis of the fluorogenic substrate DEVD-AMC in the presence or in the absence of the caspase-3 inhibitor DEVD-CHO. Data are the mean±SD of replicates of a representative experiment.

Figures 5A-D. Ang-1 stimulates Akt phosphorylation and kinase activity.

A. MVEC were incubated with Ang-1 (250 ng/ml) for 15 min and analyzed for Akt phosphorylation (serine 473, upper panel) or total Akt expression (lower panel) by Western blotting. Cells were treated as described, and cell lysates prepared for Akt kinase assays (see **B**). 20 μg of protein was separated on SDS-polyacrylamide gel electrophoresis gel (SDS-PAGE) and transferred onto a polyvinylidene difluoride membrane (Millipore). After blocking with T-PBS (PBS containing 0.2% Tween 20) containing 5% milk for 1 h, the membrane was incubated with anti-Akt antibody (Santa Cruz), phospho-specific Akt antibody (New England Biolabs). ECL (Amersham) was used for detection. **B.** Akt was immunoprecipitated from MVEC and analyzed for kinase activity using histone 2B as a substrate. (Cells were washed twice with PBS and lysed with cell lysis buffer (1% Nonidet P-40, 10% glycerol, 137

mM NaCl, 20 mM Tris-HCl, pH 7.4, 20 mM NaF, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Lysates were precleared with protein G-agarose for 30 min at 4°C, and immunoprecipitated for 2 h with anti-Akt antibodies in the presence of 2 mg/ml bovine serum albumin with or without 16 μg/ml competitor peptides (Santa Cruz). Immunoprecipitates were washed twice with cell lysis buffer, once with water, and once with kinase buffer (20 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 10 mM $MnCl_2$). Immunoprecipitated proteins were incubated in 50 μl of kinase buffer containing 2 μg of histone H2B (Roche Molecular Biochemicals) and [^{32}P-]ATP (5 μM, 10 μCi) for 30 min at room temperature. Kinase reactions were stopped by the addition of SDS sample buffer and samples subjected to Cerekenov counting and SDS-PAGE followed by autoradiography. Parallel samples were processed to confirm equal amounts of immunoprecipitated Akt. **C.** Time-dependent activation of Akt by Ang-1. MVEC were incubated with Ang-1 for increasing amount of time and Akt activation determined as above. **D.** Ang-1 induced Akt phosphorylation is blocked by soluble Tie-2 and Ang-2, but not by soluble Tie 1. MVEC were incubated with vehicle (TBS plus CHAPS), or the various indicated combinations of Ang-1 (250 ng/ml), Ang-2 (alone (2.5 μg/ml), soluble Tie 1 or Tie 2 receptors (2.5 μg/ml) for 15 min before determination of Akt phosphorylation or total Akt expression by Western blotting. Ang-1-induced Akt phosphorylation is blocked by soluble Tie-2, but not by Tie 1 or Ang-2. For all panels, data are representative of at least 3 experiments.

Figures 6A-C. Ang-1 inhibits endothelial cell apoptosis via a PI-3 kinase/Akt pathway.

A. MVEC were plated onto bacteriological dishes in serum-free media for 18h in the absence or presence of Ang-1 (250 ng/ml) or wortmannin (WM, 200 nM), before determination of apoptosis by propidium iodide staining and flow cytometry. (MVEC were plated on bacteriological dishes in serum free medium in the presence or either vehicle (TBS containing CHAPS) or Ang-1 (250ng/ml). Cells were incubated for 18hr and both floating and adherent cells were collected. To determine the number

of subdiploid cells, MVEC were fixed for 1 hour in 70% ethanol and stained with a solution containing 500 μg/ml RNAase H and 50 μg/ml propidium iodide and analyzed by using a fluorescence activated cell sorter (FACS). At least 5000 events were analyzed, and the percentage of cells in the sub-G1 population calculated.) **B.** The experimental conditions are the same as in **A**, except that MVEC were infected with adenovirus encoding β-galactosidase or dominant-negative AA-Akt for 24 h, followed by placement into suspension and treatment with Ang-1 for 18 h. For each panel, the percentage of MVEC with hypodiploid (apoptotic) DNA content is indicated. **C.** Ang-1 activates Akt in an integrin-independent manner. MVEC plated on bacteriological dishes in serum free medium were treated with vehicle or Ang-1 (250 ng/ml) in the absence or in the presence of WM (200 nM) for 15 min, and immunoblotted for phosphorylated Akt (upper panel) or total Akt (lower panel). For all panels, data are representative of 3 independent experiments.

Figures 7A-D. Ang-1 induces survivin expression via a PI-3 kinase/Akt pathway.

A. Time dependent expression of survivin RNA. Serum starved MVEC were treated with Ang-1 for the indicated time intervals and survivin, GAPDH and bcl-2 RNA expression were examined by Northern hybridization. **B.** Soluble Tie 2 prevents Ang-1 induction of survivin RNA. The experimental conditions are the same as in **A**, except that Ang-1 was preincubated in the absence or in the presence of a soluble Tie 2 receptor before addition to MVEC for 24 h and determination of survivin, GAPDH or bcl-2 RNA expression. **C.** Ang-1 stimulates survivin promoter activity. MVEC were co-transfected with plasmids encoding a promoterless luciferase cassette (pLUC-42), or a 1.2 kb survivin promoter fragment (pLUC-cyc1.2) with β-galactosidase and relative luciferase activity was determined. **D.** VEGF and Ang-1 increase survivin protein expression. HUVEC were treated with VEGF (50 ng/ml) or Ang-1 (250 ng/ml) under the various conditions tested, were harvested after 18 h and analyzed for survivin, actin and bcl-2 protein expression by Western blotting.

Numbers below the survivin panel indicate relative levels based on densitometry. For all panels, data are representative of 2-4 experiments.

Figures 8A-B. Survivin mediates the anti-apoptotic effect of Ang-1.

A. TNFα/cycloheximide. **B.** Anoikis. MVEC were transfected with GFP vector, GFP-survivin (survivin) or GFP-C84A survivin (C84A survivin), followed by treatment with TNFα (5 ng/ml) plus cycloheximide (5 g/ml) (**A**), or by plating in serum-free medium on bacteriological dishes (**B**), in the absence or in the presence of Ang-1 (250 ng/ml). Apoptosis under the various conditions tested was determined by propidium iodide staining and flow cytometry. The percentage of cells with hypodiploid DNA content quantified in the GFP-expressing population is shown in each histogram. Data are representative of 2 experiments in duplicate.

Figures 9A-C Antisense Inhibition of Survivin Expression in EC.

A. EC were transfected with the indicated increasing concentrations of control scrambled (Control) or the survivin antisense oligonucleotide (Survivin AS) followed by hybridization with cDNAs for survivin or GAPDH. Densitometric quantitation of hybridizing bands under the various conditions tested is shown in the bottom panel. **B.** EC were serum-starved for 18 h in the presence of 0.1% FCS, stimulated with VEGF (50 ng/ml) in the presence of the indicated oligonucleotide concentrations. Protein-normalized aliquots of detergent-solubilized EC extracts were immunoblotted with an antibody to survivin or β-actin followed by chemiluminescence. **C.** The experimental conditions are the same as in **B**, except that EC extracts treated with control or the survivin antisense oligonucleotide were analyzed with an antibody to bcl-2 by Western blotting. For panels **B** and **C**, molecular weight markers in kDa are indicated on the left. WB, Western blot.

Figures 10A-D. Inhibition of VEGF cytoprotection by survivin targeting.

A. EC were transfected with control or the survivin antisense oligonucleotide, treated with VEGF (50 ng/ml) and exposed to C-6 ceramide (25 μg/ml). Cell nuclei were

stained with 6.5 μg/ml 4,6- diamidino-2-phenylindole (DAPI, Sigma), 16% polyvinyl alcohol and 40% glycerol and scored morphologically for apoptosis (chromatin condensation, DNA fragmentation) using a Zeiss fluorescent microscope. Photographs of phase contrast or DAPI staining of each field are from a representative experiment out of at least three independent determinations. **B.** The experimental conditions are the same as in **A.** Data are expressed as percent of apoptosis as determined by nuclear morphology by DAPI staining, and represent the mean±SEM of three independent experiments. **C** and **D.** EC transfected with control or the survivin antisense oligonucleotide were incubated with C-6 ceramide (25 μg/ml, **C**) or the combination of TNFα (10 ng/ml) plus cycloheximide (10 μg/ml, **D**) for 12 h at 37^{0}C. Cells were analyzed for DNA content by propidium iodide staining and flow cytometry. The percentage of apoptotic cells with hypodiploid, *i.e.,* sub-G1, DNA content is indicated per each condition tested. Data are representative of an experiment of at least two independent determinations. Comparable transfection efficiencies were demonstrated by fluorescence microscopy of EC transfected with FITC-conjugated oligonucleotides.

Figures 11A-C. Modulation of Caspase Activity by Survivin Targeting.

A. The conditions are as described in Figure 2. Ceramide-treated EC stimulated with VEGF and transfected with control or the survivin antisense oligonucleotide were analyzed for caspase-3 activity by hydrolysis of the fluorogenic substrate DEVD-AMC in the presence or absence of the caspase inhibitor DEVD-CHO. Data are the mean±SD of two independent determinations. **B.** EC extracts under the various indicated conditions were analyzed for caspase-3 proteolytic cleavage with an antibody to caspase-3 by Western blotting. The positions of ~32 kDa proform caspase-3, of the intermediate product of ~24 kDa, and of active subunits of ~17 and ~19 kDa are indicated. **C.** EC extracts were analyzed for proteolytic cleavage of the caspase-3 substrate, PARP by Western blotting. Densitometric quantitation under the various conditions tested was carried out on the ~17 kDa active caspase-3 subunit (**B**) or the apoptotic ~85 kDa PARP fragment (**C**).

Figures 12A and B. Effect of survivin targeting on quiescent EC.

Starved EC were transfected with the indicated oligonucleotides and incubated in the absence (-Ceramide) or presence (+Ceramide) of ceramide. Cell extracts were analyzed for caspase-3 activity by hydrolysis of the fluorogenic substrate DEVD-AMC, in the presence or in the absence of DEVD-CHO (**A**), or by DNA content by propidium iodide staining and flow cytometry (**B**). Data are expressed as the mean±SD of three independent experiments. In **B**, the percentage of apoptotic cells with hypodiploid, sub-G1, DNA content is indicated.

Figure 13. Effect of Antisense Oligonucleotides to EC Adhesion Molecules on VEGF Cytoprotection.

The experimental conditions are as in Figure 10, except that quiescent EC were transfected with the indicated antisense oligonucleotides, stimulated with VEGF and exposed to C-6 ceramide. Cells were analyzed for nuclear morphology by DAPI staining after a 12 h culture at 37^{0}C. Data are the mean ±SEM of three independent transfection experiments.

Figure 14. Effect of survivin targeting on VEGF-induced EC migration/chemotaxis.

EC were transfected with control or the survivin antisense oligonucleotide, stimulated with VEGF and exposed to the indicated increasing concentrations of VEGF or control SPP-1 in a Boyden chamber. After a 5 h incubation at 37^{0}C, migrated cells were counted microscopically by Giemsa. Data are the mea±SEM of triplicates of a representative experiment out of three independent determinations.

Figure 15. Effect of survivin targeting on capillary formation.

EC transfected with the control or the survivin antisense oligonucleotide were cultured in collagen gels in the presence of PMA, and stabilized in the absence (None) or presence of VEGF. Three-dimensional capillary networks were analyzed by phase

contrast microscopy during a 3-d culture at 37°C. Pictures are representative of one experiment out of at least three independent determinations. Magnification x100.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

I. General Description

The present invention is based in part on the discovery that survivin is a growth factor-inducible protective gene expressed by endothelial cells during angiogenesis. Stimulation of quiescent endothelial cells with mitogens, including vascular endothelial growth factor or basic fibroblast growth factor, induced up to ~16-fold up-regulation of survivin. Mitogen stimulation rapidly increased survivin RNA expression in endothelial cells, which peaked after 6-10 h culture and decreased by 24-h. Inflammatory cytokines, tumor necrosis factor α or interleukin-1 did not induce survivin expression in endothelial cells. Formation of three-dimensional vascular tubes, *in vitro*, was associated with strong induction of survivin in endothelial cells, as compared with two-dimensional cultures. By immunohistochemistry, survivin was minimally expressed in endothelium of non-proliferating capillaries of normal skin, whereas it became massively upregulated in newly formed blood vessels of granulation tissue, *in vivo*. Recombinant expression of Green Fluorescent Protein-survivin in endothelial cells reduced caspase-3 activity and counteracted apoptosis induced by tumor necrosis factor α /cycloheximide. These findings identify survivin as a novel growth factor-inducible protective gene expressed by endothelial cells during angiogenesis.

The present invention is also based on the discovery that Ang-1 acting via the Tie-2 receptor induces phosphorylation of the survival serine/threonine kinase Akt (or protein kinase B). This is associated with up-regulation of survivin in endothelial cells and protection of endothelium from death-inducing stimuli. Moreover, a dominant negative survivin mutant negates the ability of Ang-1 to protect cells from undergoing apoptosis. The activation of anti-apoptotic pathways mediated by Akt and survivin in endothelial cells may contribute to Ang-1 stabilization of vascular structures during angiogenesis, *in vivo*.

Further, the present invention is based on the finding that an antisense oligonucleotide to the apoptosis inhibitor survivin suppressed survivin expression in endothelial cells induced by vascular endothelial cell growth factor (VEGF). In contrast, the survivin antisense oligonucleotide did not affect anti-apoptotic bcl-2 levels in endothelium. When assessed in cell death assays, antisense targeting of survivin abolished the anti-apoptotic function of VEGF against TNFα - or ceramide-induced cell death, enhanced caspase-3 activity, promoted the generation of a ~17 kDa active caspase-3 subunit, and increased cleavage of the caspase substrate, poly-ADP ribose polymerase. In contrast, the survivin antisense oligonucleotide had no effect on endothelial cell viability in the absence of VEGF. Antisense oligonucleotides to platelet-endothelial cell adhesion molecule-1 (PECAM-1, CD31), lymphocyte function-associated molecule-3 (LFA-3, CD58) or intercellular adhesion molecule-1 (ICAM-1, CD54) did not reduce the anti apoptotic function of VEGF in endothelium. When tested on other angiogenic functions of VEGF, antisense survivin targeting induced rapid regression of three-dimensional capillary networks, but did not affect endothelial cell migration/chemotaxis. These data suggest that the anti-apoptotic functions of VEGF during angiogenesis are largely mediated by the induced expression of survivin in endothelial cells. Accordingly, the present invention provides methods of modulating this pathway to increase endothelial cell viability in compensatory angiogenesis or to facilitate endothelial cell apoptosis and promote vascular regression during tumor angiogenesis.

II. Specific Embodiments

A. Survivin Molecules

1. Survivin Polypeptides

The present invention employs survivin protein, as well as allelic variants of the survivin protein, and conservative amino acid substitutions of the survivin protein. As used herein, the term "survivin protein" or "survivin" refers in part to a protein that has the amino acid sequence of human survivin. The term also includes naturally occurring allelic variants of survivin, which include naturally occurring proteins that have a slightly

different amino acid sequence than that specifically recited above. Allelic variants, though possessing a slightly different amino acid sequence than those recited above, will still have the requisite ability to inhibit cellular apoptosis. As used herein, the survivin family of proteins also refers to survivin proteins that have been isolated from organisms in addition to humans.

As disclosed above, the survivin proteins of the present invention further include conservative variants of the survivin proteins herein described. As used herein, a conservative variant refers to alterations in the amino acid sequence that do not adversely affect the ability of the survivin protein to bind to a survivin binding or signaling partner and/or to inhibit cellular apoptosis. A substitution, insertion or deletion is said to adversely affect the survivin protein when the altered sequence prevents the survivin protein from associating with a survivin binding or signaling partner and/or prevents the survivin protein from inhibiting cellular apoptosis. For example, the overall charge, structure or hydrophobic/hydrophilic properties of survivin can be altered without adversely affecting the activity of survivin. Accordingly, the amino acid sequence of survivin can be altered, for example to render the peptide more hydrophobic or hydrophilic, without adversely affecting the activity of survivin.

The allelic variants, the conservative substitution variants and the members of the survivin family of proteins, will have the ability to inhibit cellular apoptosis. Such proteins will ordinarily have an amino acid sequence having at least about 75% amino acid sequence identity with the human survivin sequence, more preferably at least about 80%, even more preferably at least about 90%, and most preferably at least about 95%. Identity or homology with respect to such sequences is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the known peptides, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and including any conservative substitutions as being homologous. N-terminal, C-terminal or internal extensions, deletions, or insertions into the peptide sequence shall not be construed as affecting homology.

Thus, the survivin proteins of the present invention include molecules having full length amino acid sequence of naturally occurring proteins; fragments thereof or peptides having a consecutive sequence of at least about 3, 5, 10, 15, or more amino acid residues of the survivin protein; amino acid sequence variants of such sequence wherein an amino acid residue has been inserted N- or C-terminal to, or within, the sequence of a naturally occurring survivin protein; amino acid sequence variants of the disclosed survivin sequence, or their fragments as defined above, that have been substituted by another residue. Contemplated variants further include those containing predetermined mutations by, *e.g.*, homologous recombination, site-directed or PCR mutagenesis, and the corresponding survivin proteins of other animal species, including but not limited to rabbit, rat, murine, porcine, bovine, ovine, equine and non-human primate species, and the alleles or other naturally occurring variants of the survivin family of proteins; and derivatives wherein the survivin protein has been covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid (for example a detectable moiety such as an enzyme or radioisotope.

The present invention also includes or employs survivin peptidomimetics. Survivin peptidomimetics are compounds that mimic the activity of survivin peptides. They are structurally similar to survivin peptides but have a chemically modified peptide backbone. Peptidomimetics may have significant advantages over polypeptide embodiments, including, for example: more economical production; greater chemical stability; enhanced pharmacological properties (half-life, absorption, potency, efficacy, *etc.*); altered specificity (*e.g.*, a broad-spectrum of biological activities); reduced antigenicity; and others.

2. Survivin Nucleic acids

The present invention also employs nucleic acid molecules or transgenes that encode survivin, and the related survivin proteins. As used herein, "nucleic acid" is defined as RNA or DNA that encodes a peptide as defined above, or is complementary to

a nucleic acid sequence encoding such peptides, or hybridizes to such a nucleic acid and remains stably bound to it under stringent conditions, or encodes a polypeptide sharing at least 75% sequence identity, preferably at least 80%, and more preferably at least 85%, with the peptide sequences. Specifically contemplated are genomic DNA, cDNA, mRNA and antisense molecules, as well as nucleic acids based on an alternative backbone or including alternative bases whether derived from natural sources or synthesized.

As used herein, "stringent conditions" are conditions in which hybridization yields a clear and detectable sequence. Stringent conditions are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015M NaCl/0.0015M sodium titrate/0.1% SDS at 50°C., or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 (g/ml), 0.1% SDS, and 10% dextran sulfate at 42°C., with washes at 42°C. in 0.2 x SSC and 0.1% SDS. A skilled artisan can readily determine and vary the stringency conditions appropriately to obtain a clear and detectable hybridization signal.

The present invention further employs fragments of the survivin encoding nucleic acid molecule. As used herein, a fragment of a survivin encoding nucleic acid molecule refers to a small portion of the entire protein encoding sequence. The size of the fragment will be determined by the intended use. For example, if the fragment is chosen so as to encode an active portion of the survivin protein, such as the C-terminal coils or the IAP motif, the fragment will need to be large enough to encode the functional region(s) of the Survivin protein.

Modifications to the primary structure itself by deletion, addition, or alteration of the amino acids incorporated into the protein sequence during translation can be made without destroying the activity of the protein. Such substitutions or other alterations

result in proteins having an amino acid sequence encoded by DNA falling within the contemplated scope of the present invention.

B. Survivin Antisense Molecules

An antisense survivin molecule is complementary to and capable of hybridizing or annealing with the RNA encoded by a survivin gene (the "sense gene"). An antisense survivin molecule is used to inhibit the expression of the survivin gene thereby inhibiting angiogenesis and preventing and treating diseases associated with angiogenesis.

Antisense nucleic acids are preferably constructed by inverting the coding region of the sense gene relative to its normal presentation for transcription to allow for transcription of its complement, hence the complementariness of the respective RNAs encoded by these DNA's. In order to block the production of mRNA produced by the sense gene, the antisense DNA should preferably be expressed at approximately the same time as the sense gene if the antisense nucleic acid is to be expressed in the cell. The timing must be approximate in the sense that the antisense RNA must be present within the cell to block the function of the RNA encoded by the sense gene. To accomplish this result, the coding region of the antisense DNA is often placed under the control of the same promoter as found in the sense gene thereby causing both to be transcribed at the same time.

For reviews of the design considerations and use of antisense oligonucleotides, see Uhlmann *et al.* (1990) and Milligan *et al.* (1993), the disclosures of which are hereby incorporated by reference.

While in principle, antisense nucleic acids having a sequence complementary to any region of the survivin gene may be useful in the angiogenesis inhibiting methods of the present invention, nucleic acid molecules complementary to a portion of the survivin mRNA transcript including the translation initiation codon are particularly preferred. Also preferred are nucleic acid molecules complementary to a portion of the survivin mRNA transcript lying within about 40 nucleotides upstream (the 5' direction) or about 40 nucleotides downstream (the 3' direction) from the translation initiation codon.

In another embodiment, antisense oligonucleotides which hybridize or anneal to at least a portion of the survivin mRNA in a cell may be used in the methods of the invention. Such oligonucleotides are typically short in length and fairly easily diffusible into a cell. Such antisense oligonucleotides include, but are not limited to, polydeoxynucleotides containing 2'-deoxy-D-ribose, polyribonucleotides containing D-ribose, any other type of polynucleotide which is an N-glycoside of a purine or pyrimidine base, or other polymers containing nonnucleotide backbones (*e.g.*, protein nucleic acids and synthetic sequence specific nucleic acid polymers commercially available) or nonstandard linkages, providing that the polymers contain nucleotides in a configuration which allows for base pairing and base stacking such as is found in DNA and RNA. They may include double- and single-stranded DNA, as well as double- and single-stranded RNA and DNA:RNA hybrids, and also include, as well as unmodified forms of the polynucleotide or oligonucleotide, known types of modifications, for example, labels which are known to those skilled in the art, "caps", methylation, substitution of one or more of the naturally occurring nucleotides with analogue, internucleotide modifications such as, for example, those with uncharged linkages (*e.g.*, methyl phosphonates, phosphorotriesters, phosphoramidates, carbamates, *etc.*) and with charged linkages or sulfur-containing linkages (*e.g.*, phosphorothioates, phosphorodithioates, *etc.*), those containing pendant moieties, such as, for example, proteins (including nucleases, nuclease inhibitors, toxins, antibodies, signal peptides, poly-L-lysine, *etc.*) and saccharides (*e.g.*, monosaccharides, *etc.*), those with intercalators (*e.g.*, acridine, psoralen, *etc.*), those containing chelators (*e.g.*, metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylating agents, and those with modified linkages (*e.g.*, alpha anomeric nucleic acids, *etc.*).

The terms "nucleoside", "nucleotide" and "nucleic acid" as used concerning survivin antisense nucleic acid molecules, include those moieties which contain not only the known purine and pyrimidine bases, but also other heterocyclic bases which have been modified. Such modifications include methylated purines and pyrimidines, acylated purines and pyrimidines, or other heterocycles. Modified nucleosides or nucleotides will

also include modifications on the sugar moiety, *e.g.*, wherein one or more of the hydroxyl groups are replaced with halogen, aliphatic groups, or are functionalized as ethers, amines, or the like.

C. Angiogenesis

Angiogenesis is the process by which new blood vessels are formed (Folkman *et al.* 1992). Thus, angiogenesis is essential in reproduction, development, and wound repair. However, inappropriate angiogenesis can have severe consequences. For example, it is only after many solid tumors are vascularized as a result of angiogenesis that the tumors begin to grow rapidly and metastasize. Because angiogenesis is so critical to these functions, it must be carefully regulated in order to maintain health. The angiogenesis process is believed to begin with the degradation of the basement membrane by proteases secreted from EC activated by mitogens such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). The cells migrate and proliferate, leading to the formation of solid endothelial cell sprouts into the stromal space, then, vascular loops are formed and capillary tubes develop with formation of tight junctions and deposition of new basement membrane.

The rate of angiogenesis involves a change in the local equilibrium between positive and negative regulators of the growth of microvessels. Abnormal angiogenesis occurs when the body loses its control of angiogenesis, resulting in either excessive or insufficient blood vessel growth. For instance, conditions such as myocardial infarction, peripheral vascular occlusion, brain ischemia, or stroke may result from the absence of angiogenesis normally required for natural healing. On the contrary, excessive blood vessel proliferation may favor tumor growth and spreading, blindness, psoriasis and rheumatoid arthritis.

Recently, the feasibility of gene therapy for modulating angiogenesis has been demonstrated. For example, promoting angiogenesis in the treatment of ischemia was demonstrated in a rabbit model and in human clinical trials with VEGF using a Hydrogel-coated angioplasty balloon as the gene delivery system. Successful transfer

and sustained expression of the VEGF gene in the vessel wall subsequently augmented neovascularization in the ischemic limb (Takeshita *et al.*, (1996); Isner *et al.*, 1996).

Alternative methods for regulating angiogenesis are still desirable for a number of reasons. For example, it is believed that native endothelial cell (EC) number and/or viability decreases over time. Thus, in certain patient populations, *e.g.*, the elderly, the resident population of ECs that is competent to respond to administered angiogenic cytokines may be limited. Moreover, while agents promoting or inhibiting angiogenesis may be useful at one location, they may be undesirable at another location. Thus, means to more precisely regulate angiogenesis at a given location are desirable.

The present invention provides a method of regulating angiogenesis by providing survivin or a modulator of survivin expression to a cell or tissue. A modulator of survivin expression is a molecule that alters the expression of survivin in a cell.

1. Methods of Inducing Angiogenesis

In one embodiment, the present invention provides methods of inducing angiogenesis. The present invention is based on the findings that survivin, an apoptosis inhibitor, is expressed by EC during angiogenesis and vascular remodeling, that Ang-1 prevents EC apoptosis by phosphorylation of Akt, and by up-regulating survivin, and that phosphorylation of Akt is required for survivin expression. Since inhibition of apoptosis may be required during vascular remodeling and angiogenesis, survivin and agents that increase the expression of survivin or the activity of survivin are useful for inducing angiogenesis. As used herein, the term "survivin activity" refers to the activities associated with survivin, for example, inhibition of apoptosis by survivin. Accordingly, survivin, Ang-1, Akt, or any other agent that increases the expression of survivin or promotes the functional activity of survivin can be used to induce angiogenesis. Administering the molecules in an amount that is effective to inhibit apoptosis may be sufficient to induce angiogenesis. Likewise, an apoptosis inhibiting amount of survivin, Ang-1, Akt, and or other agent that increases the expression of survivin would be effective in treating diseases and conditions that require inducing compensatory

angiogenesis. Examples of diseases and conditions that can be treated by these molecules include myocardial infarction, peripheral vascular occlusion, brain ischemia, and stroke.

2. Methods of Preventing Angiogenesis

In another embodiment, the present invention provides methods of inhibiting angiogenesis using agents that inhibit the expression of survivin. An example of an inhibitor of survivin expression is an antisense molecule. Alternatively, a modulator that inhibits the expression of survivin or a survivin dominant negative mutant can be used such as the C84A mutant (see Li *et al.*, (1999), which is herein incorporated by reference in its entirety). An antisense survivin molecule or an agent that inhibits the expression of survivin is useful to prevent diseases or conditions such as restenosis, vascular bypass graft occlusion, transplant coronary vasculopathy, rheumatoid arthritis, psoriasis, ocular neovascularization, diabetic retinopathy, neovascular glaucoma, angiogenesis dependent tumors, and tumor metastasis.

Agents that inhibit the functions of survivin are also useful in inhibiting angiogenesis, especially in cancerous cells to prevent metastases. Examples of such agents include, but are not limited to, survivin antibodies, inhibitors of Akt phosphorylation, and inhibitors of survivin function.

D. Methods of Delivering a Survivin Transgene or Antisense Molecule

Gene therapy is a method for delivering functionally active therapeutic or other forms of genes into targeted cells. Initial efforts of gene transfer into somatic tissues have relied on indirect means called *ex vivo* gene therapy, wherein target cells are removed from the body, transfected or infected with vectors carrying recombinant genes, and re-implanted into the body. Techniques currently used to transfer DNA *in vitro* into cells include calcium phosphate-DNA precipitation, DEAE-Dextran transfection, electroporation, liposome mediated DNA transfer or transduction with recombinant viral vectors. These transfection protocols have been used to transfer DNA into different cell

types including epithelial cells (U.S. Pat. No. 4,868,116; Morgan *et al.*, 1987), endothelial cells (WO89/05345), hepatocytes (Ledley *et al.*, 1987; Wilson *et al.*, 1990) fibroblasts (Rosenberg *et al.*, 1988; U.S. Pat. No. 4,963,489), lymphocytes (U.S. Pat. No. 5,399,346; Blaese *et al.*, 1995) and hematopoietic stem cells (Lim *et al.*, 1989; U.S. Pat. No. 5,399,346).

Direct *in vivo* gene transfer has been carried out with formulations of DNA trapped in liposomes (Ledley *et al.*, 1987), or in proteoliposomes that contain viral envelope receptor proteins (Nicolau *et al.*, 1983), and with DNA coupled to a polylysine-glycoprotein carrier complex. In addition, "gene guns" have been used for gene delivery into cells (Australian Patent No. 9068389). Lastly, naked DNA, or DNA associated with liposomes, can be formulated in liquid carrier solutions for injection into interstitial spaces for transfer of DNA into cells (WO90/11092).

Viral vectors are often the most efficient gene therapy system, and recombinant replication-defective viral vectors have been used to transduce (*i.e.*, infect) cells both *ex vivo* and *in vivo*. Such vectors include retroviral, adenovirus and adeno-associated and herpes viral vectors. Accordingly, in one embodiment the survivin transgene or survivin antisense molecule can be subcloned into an appropriate vector and transferred into a cell or tissue by gene transfer techniques discussed above.

In another embodiment, the survivin transgene or the survivin antisense molecule can be provided to the cell or tissue using a transfection facilitating composition, such as cationic liposomes containing desired polynucleotide. The desired polynucleotide is the survivin transgene or the survivin antisense molecule.

E. Methods of Delivering Survivin Expressing Cells

The present invention provides a method of delivering endothelial cells engineered to express an apoptosis inhibiting amount of survivin to a patient. Genetically engineered endothelial cells, preferably autologous, may be implanted directly into the patient, where they produce and deliver survivin. In one preferred embodiment,

autologous endothelial cells are engineered to express an apoptosis inhibiting amount of survivin.

Delivery of vertebrate cells genetically engineered to express and secrete high levels of a desired protein to a patient is well known. Typically, autologous cells derived from the patient are stably transfected with the nucleic acid encoding the desired protein. They are harvested from tissue culture dishes, placed in an implantation device, and implanted at a variety of sites including subcutaneous, intraperitoneal, intrasplenic, intraomental, inguinal, intrathecal, intraventricular, and intramuscular sites, as well as within lymph nodes or within adipose tissue.

Methods for obtaining cells stably transfected with the nucleic acid encoding survivin are discussed above.

F. Implantable Devices

As described above, the localized induction of angiogenesis using a Hydrogel-coated angioplasty balloon as the gene delivery system has been successfully demonstrated (Takeshita *et al.*, 1996; Isner *et al.*, 1996). These trials demonstrated the sustained expression of the VEGF gene in the vessel wall which subsequently augmented neovascularization in the ischemic limb. Accordingly, such delivery systems may be used to provide a survivin encoding nucleic acid molecule or transgene to a patient in need thereof. Such a system may also be used to locally deliver an agent which modulates survivin expression in contacted cells. In one preferred embodiment, both a survivin encoding nucleic acid molecule and the VEGF gene may be delivered in combination or sequentially to induce localized angiogenesis.

In another embodiment, survivin or an agent that modulates survivin expression can be provided to the tissue as an implant. The preparation of implants requires such supports suitable for being placed in contact with cells and various factors promoting the adhesion of these cells to the support if necessary, under conditions such that the different constituents present conserve their principal natural structural and functional properties. Examples of suitable implant materials are fibrous collagen and type II collagen. The

implant material is coated or impregnated with the agent that is to be provided to the cell or tissue. Other implantation device consists of a solid, unitary piece of collagen gel (a "collagen matrix") in which the cells are embedded (U.S. Pat. No. 4,485,096). Other substances, such as polytetrafluoro-ethylene (PTFE) fibers (Moullier *et al.*, 1993; WO 94/24298), may be included in the collagen implant to impart strength or other desirable characteristics to the collagen gel. Matrices may be implanted in a variety of sites. A surgical incision at the appropriate site is made, the matrix inserted, and the incision closed.

In a further embodiment, the implant can be provided to the cell or tissue as a stent. Implantable stents are small tubes of a few millimeters in diameter and a few centimers in length used to deliver therapeutic agents directly to the target site. Stents are suitable for local delivery of viral vectors encoding a survivin nucleic acid molecule or survivin inducing molecule to target sites and are often designed to be capable of degradation into products that are nontoxic to the cells of the vessel wall where they are implanted (Raiasubramanian *et al.*, 1994). Examples of biodegradable polymeric material for making stents are mixtures of poly-L-lactic acid (PLLA) and Poly-E-caprolactone (PLC) (Raiasubramanian *et al.*, 1994) and poly-beta-hydroxybutanoic acid (US Patent No. 5,935,506). The stents can be impregnated with a survivin nucleic acid molecule, a survivin antisense molecule, or an agent that induces survivin expression to be delivered and surgically implanted at the target site.

G. Pharmaceutical Composition and Methods of Delivery of Agents

The agents that promotes or inhibits angiogenesis can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. For example, as a means of blocking angiogenesis in tumor cells, a survivin inhibiting agent such as a survivin antibody, an inhibitor of survivin expression

or function, or an inhibitor of Akt phosphorylation, is administered systemically or locally to the individual being treated. Alternatively, as a means of promoting angiogenesis, an agent such as survivin itself, Ang-1, Akt, or any other agent that increases the expression of survivin or induces the function of survivin is administered systemically or locally to the individual. As described below, there are many methods that can readily be adapted to administer such agents.

The present invention contemplates compositions containing one or more agents that promotes or inhibits angiogenesis. While individual needs vary, a determination of optimal ranges of effective amounts of each component in the composition is within the skill of the art. Typical dosages comprise 0.1 to 100 mg/kg body wt. The preferred dosages comprise 0.1 to 10 mg/kg body wt. The most preferred dosages comprise 0.1 to 1 mg/kg body wt.

In addition to the pharmacologically active agent, the compositions of the present invention may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action.

Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

As described above, the pharmaceutical formulations for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient.

Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

In practicing the methods of this invention, the agents that induce or inhibit angiogenesis may be used alone or in combination, or in combination with other therapeutic or diagnostic agents. In certain preferred embodiments, the agents may be coadministered along with other compounds typically prescribed for these conditions according to generally accepted medical practice, such as chemotherapeutic agents.

In one aspect, the present invention contemplates the use of agents that modulate survivin expression or function in combination with an immunomodulatory agent in an immunosuppressive therapy, such as those used to treat patients who have received a transplant or graft. Transplants of healthy organs or cells into a patient suffering from a disease are often rejected by the body due to an immune response initiated in response to the foreign tissue or cells. At present, the only method to inhibit this immune response is to administer chronic nonspecific immunosuppression agents. Agents that modulate survivin expression or function can inhibit or induce angiogenesis depending on the condition, and immunomodulatory agents can suppress undesirable immune response. The combination would facilitate the recovery of patients who have undergone transplantation.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

Example 1

Materials and Methods

Cells and Cell Culture.

Human umbilical vein EC were maintained in M199 medium supplemented with 20% fetal calf serum (FCS), 50 μg/ml Endothelial Cell Growth Supplement (ECGS), 100 μg/ml heparin, 100 μg/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies, Grand Island, NY), in 5% CO_2 at 37°C. Bovine aortic EC were isolated and maintained in culture as described by De Luca *et al.* (1994). Subconfluent EC were rendered quiescent by a 24 h-culture in M199 plus 0.1% FCS. Cells were detached with 0.05% trypsin/0.02% EDTA, seeded in C6-well plates (Costar Corp., New Bedford, MA), grown to 70% confluency and used between passage 2 and 3.

Modulation of Survivin Expression in EC.

Quiescent sub-confluent EC were incubated with VEGF (Collaborative Biomedical Products, Bedford, MA; 10-100 ng/ml), basic fibroblast growth factor (bFGF Calbiochem Corp., La Jolla, CA; 5 ng/ml), 10% FCS, or recombinant IL-1 (R&D, Minneapolis, MN; 2 ng/ml, 200 U/ml) or TNFα (10ng/ml, Endogen, Woburn, MA) for 14 h at 37°C in M199 plus 0.1% FCS. Cells were washed, harvested by trypsin/EDTA and extracted in 4% SDS plus protease inhibitors. Protein normalized aliquots of cell extracts were electrophoresed on a 13.5% SDS polyacrylamide gel, transferred to nylon membranes (Millipore, Corp.) for 1 h at 1 A, and immunoblotted with 1 μg/ml of a rabbit antibody to survivin followed by chemiluminescence (Amersham, Arlington Heights, IL). 18 Samples were analyzed for equal protein loading by immunoblotting with a mouse antibody to β-actin. For Northern hybridization, serum-deprived EC were stimulated with 100 ng/ml VEGF and harvested at increasing time intervals between 1.5-24 h culture at 37°C. Total RNA was extracted using the TRI Reagent (10^6 cells/0.2 ml, Molecular Research Center, Cincinnati, OH), and further processed for Northern hybridization with a ^{32} Pα-dCTP-random-primed labeled survivin cDNA or control β-actin probe, as described (Ambrosini *et al.* 1997).

Three-Dimensional EC Culture.

EC were suspended at a density of $3x10^6$ /ml in a liquefied matrix of rat-tail type I collagen (1.5 mg/ml) and human plasma-derived fibronectin (0.15 mg/ml) in M199, pH 7.5. One ml of the EC suspension was transferred into each well of rat-tail type I-coated C6 wells, and warmed to 37°C to allow polymerization of the matrix. After a 24-h incubation at 37°C in M199 plus 20% FCS, 50 µg/ml ECGS, 100 µg/ml heparin, 100 µg/ml penicillin and 100 µg/ml streptomycin, the three dimensional culture was placed in OCT and paraffin-embedded for immunohistochemical analysis. Alternatively, two- or three-dimensional EC cultures were homogenized in a tissue grinder and immunoblotted for survivin expression. During the incubation period, EC throughout the gel were observed to elongate and form multicellular tubular structures, as described (Sierra-Honigman *et al.*, 1998).

Immunohistochemistry.

Four skin biopsies, containing granulation tissue or normal, non-inflamed skin by hematoxylin-eosin staining were collected from the archives of Yale-New Haven Hospital. Five µm sections were prepared from paraffin-embedded tissues, deparaffinized in xylene, and rehydrated in graded alcohol with quenching of endogenous peroxidase in 2% H_2O_2 in methanol. Immunolocalization of survivin was carried out as described previously (Ambrosini *et al.*, 1997) after antigen retrieval by pressure cooking for 5 min in 0.01 M citrate buffer, pH 6.0. Binding of the primary antibody was revealed by addition of 3,3'-diaminobenzidine, or, alternatively, 3-amino-9-ethylcarbazol (AEC, Vector), as a substrate. Control experiments were carried out in the absence of primary antibody, or in the presence of preimmune rabbit IgG.

EC Protection by Survivin.

The cDNA of wild type survivin (Ambrosini *et al.*, 1997) was inserted in frame in the *Eco*RI site of Green Fluorescence Protein (GFP)-encoding plasmid, pEGFPc1 (Clontech, San Francisco). The correct orientation and reading frame of pEGFPc1 fusion

plasmid were confirmed by DNA sequencing. Bovine aortic EC were seeded in C6-well plates at 40-50% confluency and transfected with GFP-vector or GFP-survivin by lipofectin for 6 h at 37°C. After removal of the DNA-lipid mixture, the EC monolayer was placed in complete growth medium for 35 h at 37°C, and incubated with 5 ng/ml TNFα plus 5 μg/ml cycloheximide for additional 8h at 37°C. Cells (floaters plus attached cells) were fixed in 70% ethanol, stained with 10 μg/ml propidium iodide plus 100 μg/ml RNAse A and 0.05% Triton X-100 in PBS, pH 7.4, and GFP-expressing cells were analyzed for DNA content by flow cytometry. In other experiments, bovine EC transfected with GFP-vector or GFP-survivin were treated with control medium or 5-10 ng/ml TNFα plus 10 g/ml cycloheximide for 8-h at 37°C. Cells were harvested, and analyzed for caspase-3 activity by hydrolysis of the fluorogenic substrate Ac-DEVD-AMC (N-Acetyl- Asp-Glu-Val-Asp-aldehyde, Pharmingen, San Diego, CA), in the presence or in the absence of the caspase-3 inhibitor Ac-DEVD-CHO. Fluorescence emissions were quantitated on a spectrofluorometer with excitation wavelength of 360 nm and emission of 460 nm.

Example 1.1

Mitogen-Stimulated Induction of Survivin in EC.

Expression of ~16.5 kD endogenous survivin in quiescent, serum-deprived endothelium was minimally detectable by immunoblotting (Figure 1A), in agreement with previous observations (Ambrosini *et al.* 1997). EC stimulation with serum, or specific mitogens, VEGF or bFGF, resulted in an 8-16-fold up regulation of survivin expression, by immunoblotting (Figure 1A). Survivin induction by VEGF was concentration-dependent and maximal at ~50 ng/ml (Figure 1B). EC stimulation with cytokines TNFα or IL-1 did not increase survivin expression, which was reduced below background levels of untreated cells (Figure 1A). In control experiments by flow cytometry, TNFα or IL-1 stimulated strong up regulation of intercellular adhesion molecule-1 in EC, whereas VEGF was ineffective (not shown). By Northern hybridization, a main 1.9-kb survivin message and a fainter 3.4-kb survivin transcript

were minimally detected in quiescent EC (Figure 1C). VEGF treatment resulted in rapid up regulation of survivin RNA in EC, in a response that peaked 6- to 10-h after stimulation, and decreased to approach background levels 24 h after treatment (Figure 1C).

Example 1.2

Survivin Expression in Three-Dimensional EC Cultures.

Survivin was expressed at very low levels in two-dimensional EC cultures, by immunohistochemistry (Figure 2A). In contrast, formation of three-dimensional vascular tubes in collagen/fibronectin matrix resulted in strong expression of survivin in EC (Figure 2B). No staining of three-dimensional EC cultures was observed with control non-binding antibody (Figure 2C). By immunoblotting, a prominent ~16.5 kD survivin band was prominently induced in EC extracts of three-dimensional vascular tubes, as compared with two-dimensional EC cultures (Figure 2D).

Example 1.3

Survivin Expression in Proliferating EC, *In Vivo*.

In four out of four cases, survivin was strongly expressed in the cytoplasm of EC of newly formed capillaries of skin granulation tissue, by immunohistochemistry (Figure 3A). Abundant expression of survivin was also demonstrated in EC of large vessels of granulation tissue at the dermis/hypodermis junction (Figure 3C). In contrast, no staining of granulation tissue was observed in the absence of primary antibody (Figure 3B, D), or with control preimmune antibody (not shown). Analysis of non-proliferating capillaries of non-inflamed normal skin revealed minimally detectable expression of survivin in EC (Figure 3E), as compared with control staining with preimmune IgG (Figure 3F).

Example 1.4

Anti-Apoptotic Effect of Survivin in EC.

Treatment with TNFα/cycloheximide induced EC apoptosis and generation of an hypodiploid population by propidium iodide staining and flow cytometry (Figure 4A). Expression of GFP-survivin inhibited TNFα-induced apoptosis in EC, and reduced the percentage of hypodiploid cells to control levels of untreated cultures (Figure 4A). In contrast, transfection of GFP-vector alone did not affect TNFα-induced EC apoptosis (Figure 4A). Moreover, expression of GFP-survivin in EC strongly inhibited caspase-3 activity in TNFα-treated EC, as determined by DEVD hydrolysis, whereas GFP-vector alone was ineffective (Figure 4B). In control experiments, preincubation of TNFα-treated EC extracts with the caspase-3 inhibitor DEVD-CHO abrogated DEVD hydrolysis (Figure 4B).

Example 2

Materials and Methods

Example 2.1

Effect of Ang-1 on Akt.

Bovine lung microvascular endothelial cells (MVEC, Vectek, Albany, NY) were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (FBS), L-glutamine and antibiotics (penicillin and streptomycin). Cells (up to passage 12) were used for the experiments; cultures had typical cobblestone morphology and stained uniformly for von Willebrand factor, as assessed by indirect immunofluorescence.

A recombinant form of Ang-1 was used in all of the experiments. This form of Ang-1 differs from the native Tie2 ligand in that it possesses a modified NH2-terminal sequence and a mutation in Cys245 that make it easier to produce and purify.

Microvascular endothelial cells (MVEC) were treated with Ang-1. Changes in the anti-apoptotic serine/threonine kinase, Akt (or protein kinase B) were analyzed. Stimulation of MVEC with Ang-1 increased Akt phosphorylation on serine 473 (Fig. 5A), threonine 308 (not shown) and up-regulated Akt kinase activity (Fig. 5B), in a

reaction suppressed by the PI3 kinase inhibitor, wortmannin (WM; Fig. 5B). Ang-1 stimulated Akt phosphorylation in a time-dependent manner with maximal activation occurring within 15-30 min, and sustained phosphorylation lasting for up to 2 h (Fig. 5C). Ang-1 stimulated phosphorylation of Akt on Ser 473 was antagonized by preincubation of Ang-1 with soluble Tie 2 receptor, but not by incubation with soluble Tie1 receptor bodies (5D). In addition, Ang-1 induced Akt phosphorylation was partially blocked by the physiological antagonist of Ang-1, angiopoeitin-2 (Ang-2; Maisonpierre *et al.*, 1997). Interestingly, Ang-2 alone weakly activated Akt in MVEC. Therefore, Ang-1 via the Tie 2 receptor stimulates Akt activation through a PI-3 kinase dependent mechanism.

Example 2.2

Effect of Ang-1 on Endothelial Cell Apoptosis Induced by Detachment from the Matrix, *i.e.* Anoikis (Frisch *et al.* 1997)

MVEC in serum free media and plated onto petri dishes for 18 h underwent extensive apoptosis as determined by appearance of a hypodiploid cell population (~25% versus 2% of control, adherent cultures) by propidium iodide staining and flow cytometry (Fig. 6A). Incubation of MVEC cultured under these conditions with Ang-1 inhibited apoptosis by 75%, in a reaction reversed by WM (Fig. 6A). To examine if Akt was required for Ang-1 cytoprotection, MVEC were infected with adenoviral β-galactosidase or activation deficient Akt (AA-Akt; Fujio *et al.*, 1999) and determined the degree of apoptosis. Transduction of MVEC with AA-Akt abrogated the cytoprotective effect of Ang-1 against anoikis, whereas a control adenovirus encoding β-galactosidase was ineffective. MVEC were infected with 100 MOI of adenovirus containing the β-galactosidase, HA-tagged activation deficient phosphorylation mutant Akt (AA-Akt, Fujio *et al.*, 1999). After 4 hr, the virus was removed and cells left to recover overnight in complete medium. In preliminary experiments with the β-galactosidase virus, these conditions were optimal for infecting 95% of the cultures. Infected cells were plated in bacteriological dishes for apoptosis experiments or lysed in buffer for immunoblotting.

WM also prevented Akt phosphorylation on Ser 473 induced by Ang-1 in suspended endothelial cells (Fig. 6B). Collectively, these data indicate that Ang-1 mediates endothelial cell protection through an integrin independent, PI-3-kinase /Akt-dependent pathway.

Example 2.3

The Potential Link between Ang-1 and Two Known Anti-Apoptotic Genes, Survivin and Bcl-2 (Ambrosini *et al.* 1997 and Gerber *et al.* 1998).

Treatment of MVEC with Ang-1 rapidly induced a time-dependent increase in survivin RNA levels (Li *et al.,* 1998), which peaked 12 h after stimulation and remained sustained for up to 24 h (Fig 7A). In contrast, Ang-1 did not up-regulate bcl-2 RNA expression in MVEC (Fig. 5A). Consistent with a receptor-mediated response, preincubation of Ang-1 with soluble Tie-2 receptor abolished Ang-1 induction of survivin RNA in MVEC (Fig. 7B). When MVEC were transfected with a survivin-luciferase promoter construct (Li *et al.,* 1999a), Ang-1 stimulated a 3-7-fold up-regulation of survivin transcriptional activity, which persisted for up to 24 h after stimulation (Fig. 7C). VEGF or Ang-1 strongly induced expression of survivin protein in HUVEC, an effect abrogated by WM, or by transduction with AA-Akt (Fig. 7D). Human umbilical vein endothelial cells (HUVEC) were used in these experiments due to greater sensitivity of the survivin antibody with human survivin. HUVEC were isolated from umbilical veins and cultured on gelatin coated tissue culture flasks in M199 containing 20% fetal bovine serum (FBS), 50 μg/ml EC growth supplement (ECGS, a commercial preparation that contains mainly acidic fibroblast growth factor), 100 μg/ml porcine heparin, 10U/ml penicillin and 100 μg/ml streptomycin. Two to three individual donors were pooled at passage one and used up to passage three. Cultures had typical cobblestone morphology and stained uniformly for von Willebrand factor, as assessed by indirect immunofluoresence. Identical results were obtained using MVEC.

In contrast, VEGF weakly induced bcl-2 protein expression in MVEC, whereas Ang-1 was ineffective (Fig. 7D). These data demonstrate that distinct angiogenic factors,

Ang-1 and VEGF, stimulate survivin expression in endothelial cells via a PI-3 kinase/Akt dependent mechanism.

Example 2.4

Induced Survivin Expression Mediates the Anti-Apoptotic Function of Ang-1

MVEC was transfected with cDNAs containing green fluorescent protein (GFP) fused to wild-type-survivin (GFP-survivin), or to a dominant negative Cys^{84}-->Ala survivin mutant (GFP-C84A survivin). Cytoprotection in response to apoptosis-inducing stimuli (Li *et al.*, 1999b) was determined. Fusion of survivin with GFP does interfere with its biological activity (Li *et al.*, 1999b). The survivin-GFP (Cys84-Ala) construct is a mutation in the BIR1 domain. It is targeted to the mitotic spindle similar to endogenous survivin but is devoid of its anti-apoptotic function.

Treatment with Ang-1, or expression of GFP-survivin, alone or in combination with Ang-1, suppressed the appearance of MVEC with hypodiploid DNA content induced by TNFα/cycloheximide or by anoikis (Figs. 8A and B). In contrast, transfection of MVEC with GFP-C84A survivin abrogated the cytoprotective effect of Ang-1 against TNFα/cycloheximide- or anoikis-induced cell death (Figs. 8A and B). These data identify survivin as a novel PI-3-kinase, Akt-dependent target gene for Ang-1, and demonstrate that survivin is necessary for the anti-apoptotic effect of Ang-1.

Example 3

Materials and Methods

Survivin Mediated *In Vivo* Angiogenesis in Animal Model

Plasmid DNA

DNA encoding survivin is assembled into a mammalian expression vector containing the cytomegalovirus promoter (Takeshita *et al.*, 1996). The biologic activity of survivin obtained from cells transfected with this construct, phSurvivin, is confirmed before performing arterial gene transfer. The plasmid pGSVLacZ containing a nuclear-targeted -galactosidase sequence coupled to the simian virus 40 early promoter is used

for control transfection experiments (Takeshita *et al.*, 1996).

Animal Model

Animal studies are performed in an animal model of hindlimb ischemia (Takeshita *et al.*, 1996). Rabbits weighing 4 to 4.5 kg are anesthetized with ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) after premedication with xylazine (2.5 mg/kg). A longitudinal incision is performed in one limb, extending inferiorly from the inguinal ligament to a point just proximal to the patella. Through this incision, the femoral artery is dissected free along its entire length using a surgical loupe; all branches of the artery are also dissected free. After dissection of the popliteal and saphenous arteries, the external iliac arter as well as all of the above arteries are ligated. Finally, the femoral artery is completely excised from its proximal origin as a branch of the external iliac artery, to the point distally at which it bifurcates into the saphenous and popliteal arteries. Once the femoral artery is excised, thrombotic occlusion of the external iliac artery extends retrograde to its origin from the common iliac artery. Consequently, blood supply to the distal limb is dependent on the collateral arteries, which may originate from the internal iliac artery. This operative procedure results in severe limb ischemia (Takashita *et al.*, 1996). Postoperative analgesia (levorphanol tartrate 60 mg/kg) is administered subcutaneously as required and prophylactic antibiotics (enrofloxancin 2.5 mg/kg) are administered subcutaneously for 5 days.

Percutaneous Arterial Gene Transfer

An interval of 10 days between the time of surgery and gene transfer is allowed for postoperative recovery of the rabbits and development of endogenous collateral vessels. At 10 days postoperatively (Day 0), a baseline angiogram is performed. The internal iliac artery of the ischemic limb of a number of animals is transfected with phSurvivin percutaneously using a 2mm hydrogel-coated balloon catheter (Slider™, Boston Scientific, Watertown massachusetts). The angioplasty balloon is prepared (*ex vivo*) by advancing the deflated balloon through a 5 Fr. Teflon sheath (Boston Scientific);

applying the solution of plasmid DNA from a conventional pipette to the 20 μm layer of hydrogel coating the external surface of the inflated balloon; and finally, deflating the balloon, retracting same into the protective sheath, and re-inflating the balloon to prevent backflow of blood into the sheath (and onto the coated balloon) after introduction into the circulation. The sheath and angioplasty catheter are then introduced via the right carotic artery and advanced to the lower abdominal aorta using a 0.014-inch guide-wire (Hi-Torque Floppy II™; Advanced Cardiovascular System, Temecula, California) under fluoroscopic guidance. The balloon catheter is then advanced into the internal iliac artery of the ischemic limb, inflated for 1 minute at 4 to 6 atmospheres, deflated and withdrawn. An identical protocol is used to transfect the internal iliac arter of control animals with the plasmid pGSVLacZ. Heparin is not administered at the time of transfection or angiography.

RT-PCR

Gene expression is evaluated at the mRNA level by RT-PCR in rabbits in which the iliac artery was transfected using the hydrogel balloon catheter, as described above. Transfected arterial segments are obtained at 7, 14, 21, and 30 days posttransfection. Remote tissues such as brain, heart, liver, lung, spleen, testes, are also retrieved ≤7 days posttransfection for analysis of survivin mRNA. Total cellular RNA is isolated using TRI reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions. Extracted DNA is treated with DNase I (0.5 μl, 10 U/μl, Rnase-free, Message Clean kit; GenHunter, Boston, Massachusetts) at 37°C for 30 minutes to eliminate DNA contamination. The yield of extracted RNA is determined spectrophotometrically by ultraviolet absorbance at 260 nm. To check that the RNA is not degraded and that the ribosomal bands are intact, each RNA sample is subjected to 1% nondenaturing mini-agarose gel electrophoresis. Each RNA sample is used to make cDNA in a reaction containing deoxynucleotides, RNasin (Promega, Madison, Wisconsin), random hexanucleotide primers (Promega), and Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, Maryland). Reactions are

incubated at 42°C for 1 hour, then at 95°C for 5 minutes to terminate the reaction. The PCR amplification is performed for 30 cycles at 94°C for 20 seconds, ending with 5 minutes at 72°C. Oligonucleotide primers selected from a specific region of the nucleic acid encoding survivin is used to amplify that region or fragment of survivin. RT-PCR products are analyzed by 2% agaose gel electrophoresis.

Transfection Efficiency

To assess the efficiency of *in vivo* arterial gene transfer in the animal model, LacZ-Tf arteries are harvested on Day 5, and β-galactosidase activity is determined by incubation with 5-bomo-4-chloro-3-indolyl-β-D-galactoside chromogen (X-Gal; Sigma Chemical Company, St. Louis Missouri) as previously described (Takeshita *et al.*, 1996). After staining with X-Gal solution, tissues are paraffin-embedded, sectioned, and counterstained with hematoxylin-eosin. Nuclear localized β-galactosidase expression of the plasmid pGSVLacZ could not result from endogenous β-galactosidase activity; accordingly, histochemical identification of β-galactosidase within the cell nucleus is interpreted as evidence for successful gene transfer and gene expression. Cytoplasmic or other staining is considered nonspecific for the purpose of the present study.

Evaluation of Angiogenesis in the Ischemic Limb

Development of collateral vessels in the ischemic limb is serially evaluated by calf blood pressure measurement and internal iliac arteriography immediately before transfection (Day 0) and again on Day 30 posttransfection. In addition to these two parameters, limb blood flow and capillary density in the ischemic limb muscles are evaluated on Day 30 posttranfection.

Calf Blood Pressure Ratio

Calf blood pressure is measured in both hindlimbs using a Doppler flowmeter (model 1059, Parks Medical Electonics, Aloha, Oregon) immediately before transfection (Day 0) as well as on Day 30. On each occasion, the hindlimbs are shaved and cleaned,

the pulse of the posterior tibial artery is identified using a Doppler probe, and the systolic blood pressure in both limbs is determined using standard techniques (Takeshita *et al.*, 1996). The calf blood pressure ratio is defined for each rabbit as the ratio of systolic pressure of the ischemic limb to systolic pressure of the normal limb.

Selective Iliac Angiography

Selective internal iliac arteriography is performed on Day 0 (immediately before transfection) and again on Day 30 posttransfection (Takeshita *et al.*, 1996). A 3 Fr. Infusion catheter (Tracker-18, Target Therapeutic, San Jose, California) is introduced into a common carotid artery through a small cutdown and advanced to the internal iliac artery of the ischemic limb using 0.014 inch guidewire (Hi-torque floppy II) under fluoroscopicguidance. The tip of catheter is positioned in the internal iliac artery at the livel of the interspace between the seventh lumbar and the first sacral vertebrae. After intraarterial injection of 0.26 mg of nitroglycerin, 5 ml of nonionic contrast media (Isovue-370, Squibb Diagnostics, New Brunswick, New Jersey) is injected using an automated angiographic injector (Medrad, Pittsburgh, Pennsylvania) programmed to reproducibly deliver a flow rate of 1ml/second. Serial angiographic images (1 per second for 10 seconds) are then recorded on 105-mm spot film. Morphometric angiographic analysis of collateral vessel development is performed using a grid overlay comprised of 2.5-mm circles arranged in rows spaced 5 mm apart. The overlay is applied to the 4-second angiogram recorded at the level of the medial thigh. A defined area is chosen in which the number of contrast-opacified arteries crossing over circles as well as the total number of circles encompassing the medial thigh area are counted in single blind fashion. An angiographic score is calculated for each film as the ratio of crossing opacified arteries divided by the total number of circles in the defined area of the ischemic thigh.

Blood Flow Measurement

A 0.018-inch guidewire with a 12-MHz piezoelectric transducer at the distal tip (FloMap: Cardiometrics, Mountainview, California) is used to measure blood flow

velocity (Takeshita *et al.*, 1996). The Doppler wire records a real-time spectral analysis of the Doppler signal from which the average peak velocity (APV, temporal average fo the instantaneous peak velocity waveform) is calculated and displayed on line. The wire is advanced through the 3 Fr. infusion catheter positioned at the origin of the common iliac artery to the proximal segment of the internal iliac artery supplying the ischemic limb. A stabilized velocity of 2 minutes before recording resting APV is required. Maximum APV is recorded after bolus injection of papaverine (Sigma Chemical Company), 2mg/0.4 ml saline, via the infusion catheter. The Doppler wire is then pulled back from the internal iliac artery and readvanced to the iliac artery of the normal limb; the distal tip of the 3 Fr. infusion catheter is repositioned at the origin of the common iliac artery. Blood flow velocity is again recorded at rest and after papaverine injection.

After completing all Doppler measurements, the 3 Fr. infusion catheter is redirected to the proximal segment of the internal iliac artery of the ischemic limb, and selective internal iliac angiography is performed as described. The angiographic luminal diameter of the internal iliac artery in the ischemic limb and of the external artery in the normal limb are determined using an automated edge-detection system (Quantum 2000l; QCS, Ann Arbor Michigan) as described. The film selected for analysis is scanned with a high resolution video camera, and the signal produced by the video camera is digitized and displayed on a video monitor (Laser Scan; ImageComm, Santa Clara, California). Center lines are traced manually for a 10-mm segment beginning immediately distal to the tip of the Doppler wire. The contours are subsequently detected automatically on the basis of the weighted sum of first and second derivative functions applied to the digitized brightness information. The vascular diameter is then measured at the site of the Doppler sample volume (5 mm distal to the wire tip). Cross Sectional area is calculated assuming a cirular lumen.

Doppler-derived flow is calculated as $Q_D = (d^2/4)(0.5 \text{ X APV})$ where Q_D= Doppler-derived time average flow, d =vessel diameter, and APV = time average of the spectral peak velocity. The mean velocity is estimated as 0.5 X APV by assuming a time-averaged parabolic velocity profile across the vessel. Angiographic luminal

diameter measurements from the angiogram recorded immediately before Doppler recording are used for calculation of rest and maximum flow.

Capillary to Muscle Fiber Ratio

The effect of survivin gene transfer upon anatomic evidence of collateral artery formation is further examined by identifying capillaries in light microscopic sections taken from the ischemic hindlimbs (Takeshita *et al.* 1996). Tissue specimens are obtained as transverse sections from the ischemic hindlimbs at the time of death (Day 30 posttransfection). Muscle samples are embedded in optimal cutting temperature compound (Miles, Elkhart, Indiana) and snap-frozen in liquid nitrogen. Multiple frozen sections (5 μm in thickness) are then cut from each specimen on a cryostat (Miles) so that the muscle fibers are oriented in a transverse fashion, and two sections are then placed on glass slides. Tissue sections are stained for alkaline phosphatase using an indoxyl-tetrazolium method to detect capillary endothelial cells (Takeshita *et al.* 1996) and then counterstained with eosin. To ensure that analysis of capillary density is not overestimated because of muscle atrophy or underestimated because of interstitial edema, capillaries identified at necropsy are evaluated in relation to muscle fibers; a total number of 20 different fields is randomly selected and the number of capillaries and muscle fibers are counted under a 20X objective to determine the capillary to muscle fiber ratio.

Evaluation of Tissue Sites Remote from the Ischemic Limb

Fourteen rabbits are selected at random from those undergoing survivin arterial gene transfer. Tissue sections are systematically retrieved from gonads, liver, heart, lung , brain, and contralateral (nontransfected) lower limb skeletal muscle and examined by light microscopy for evidence of neoangiogenesis as well as evidence of immune-related inflammtory cell infiltrates.

Morphometric Analysis of the Site of Gene Transfer.

Representative tissue sections are harvested on Day 30 from the site of gene transfer in a number of rabbits selected at random. These included a few transfected with phSurvivin and a few transfected with LacZ. In each case, the site of gene transfer is identified by the fact that gene transfer is performed at the origin of the internal iliac artery; accordingly, an arterial segment approximately 5 mm in length is retrieved from the origin of this artery just distal to the bifurcation of the common iliac artery. The section is stained with hematoxylin and eoxin and then morphometrically evaluated by light microscopy for intimal and medial thickness, from which the intima to media ratio is derived (Takeshita *et al.*, 1996).

Example 3.1

Survivin Mediated *In Vivo* Angiogenesis

Plasmid DNA

Plasmid phSurvivin consists of a eucaryotic pUC 118 expression vector into which cDNA encoding survivin has been inserted. A 763 basepair cytomegalovirus promoter/enhancer is used to drive Survivin expression. The PUC 118 vector includes an SV40 polyadenylation sequence, an *Escherichia coli* origin of replication, and the β-lactamase gene for ampicillin resistance. The plasmid is prepared from cultures of phSurvivin transformed *E coli*, purified with a Qiagen-tip 2500 column, precipitated with isopropanol washed with 70% ethanol, and dried on a Speed Vac. The purified plasmid is reconstituted in sterile saline, stored in vials, and pooled for quality control analyses (absorbance at wavelengths of 260 and 280 nm to document ratio between 1.75 and 1.85; limulus amoebocyte lysate gel-clot assay [Bio-Whittaker] to establish bacterial endotoxin levels below 5 endotoxin units per kg bodyweight; microbial cultures; southern blot for level of contaminating genomic *E coli* DNA; and ethidium bomide staining after agarose-gel electrophoresis to confirm that over 90% of the nucleic acid was in the closed, circular supercoiled form). To confirm the identity of the prepared plasmid, the survivin coding region from each pooled batch is resequenced (Applied Biosystem 373A).

Percutaneous Arterial Gene Transfer

Arterial gene transfer is performed on a patient with an ischemic leg.

Arterial gene transfer is done with a hydrogel-coated balloon-angioplasty-catheter (Boston Scientific). A sterile pipette is used to apply 2000 g plasmid DNA at 10.3 μg/μl in 194.2 μl sterile saline to external hydrogel coat of the inflated angioplasty balloon. The balloon is deflated, retracted into a protective sheath reinflated to 2280 mm Hg, and advanced along with the sheath over a 45.7 mm guidewire under floroscopic guidance to the site of gene transfer. The balloon is then deflated, the sheath retracted, and the balloon reinflated at nominal pressures for 4-5 min. The balloon is deflated, all catheters and wires removed, and a final angiogram recorded to ensure satisfactory patency of the site.

Intravascular ultrasound is done immediately before gene transfer to show that the intended site, the distal popliteal artery is free of atherosclerotic plaque that might compromise transfection efficiency (Isner *et al.*, 1996). Repeat ultrasound at 4 weeks and 12 weeks after gene transfer is done to ensure that there is no neointimal thickening resulting from inflation of the hydrogel-coated angioplasty-balloon-catheter.

Digital substraction angiography is performed 4 weeks after gene therapy, and magnetic resonance angiography is performed 4 and 12 weeks after gene therapy. Both are performed to detect gene transfer promoted angiogenesis.

Example 4

The preservation of vascular homeostasis during inflammation, immune response and transplant accommodation depends on the ability of endothelial cells (EC) to continuously counteract a cellular suicide program, *i.e.* apoptosis (Karsan *et al.*, 1996). This process involves a sequential cascade activation of intracellular cysteine proteases, *i.e.*, caspases, initiated by ligation of cell surface death receptors or by cytoplasmic assembly of cell death initiators, *i.e.*, apoptosome, after mitochondrial damage (Hengartner, 2000). Inhibition of EC apoptosis is also an obligatory prerequisite of angiogenesis, in which multiple receptor-ligand interactions at the EC surface stimulate

proliferation, migration and remodeling of EC to generate new vascular networks (Risau, 1997). In this context, antibody or adenoviral targeting of critical angiogenesis regulators, including vascular endothelial cell growth factor (VEGF) (Alon *et al.,* 1995; Yuan *et al.,* 1996), or the angiopoietin-1 (Ang-1) receptor, Tie-2 (Lin *et al.*, 1998), resulted in involution of vascular networks accompanied by morphological and biochemical hallmarks of EC apoptosis. In addition to survival signals mediated by adhesion molecule, *i.e.*, integrin, engagement (Ruegg *et al.,* 1998; Isik *et al.,* 1998), and activation of the phophoinositide 3/Akt pathways (Fujio *et al.,* 1999; Gerber *et al.,* 1998b), angiogenesis has been associated with *de novo* expression of an heterogeneous set of anti-apoptotic "protective genes" in the endothelium (Bach *et al.*, 1997), some of which become induced via NF-κB signaling (Stehlik *et al.*, 1998). Specifically, stimulation of EC angiogenesis by VEGF or Ang-1 resulted in up-regulation of anti-apoptotic bcl-2 and A1 molecules (Gerber *et al.*, 1998a; Nor *et al.*, 1999) and expression of Inhibitor of Apoptosis (IAP) proteins (Devereaux *et al.*, 1999), survivin and XIAP (O'Connor *et al.,* 2000a; Tran *et al.,* 1999; Papapetropoulos *et al.*, 2000).

In the following Examples, an antisense targeting strategy was used to identify the relative contribution of survivin to the anti-apoptotic function of VEGF in endothelium.

Materials and Methods

EC Culture.

Human umbilical vein EC were maintained in M199 medium containing 20% fetal calf serum (FCS), 50 μg/ml endothelial cell growth supplement (ECGS), 100 μg/ml heparin, 100 μg/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies, Grand Island, NY) in 5% CO_2 at 37°C, as described by O"Connor *et al.* (2000). Subconfluent EC were rendered quiescent by a 18 h culture in M199 plus 0.1% FCS. Cells were detached with 0.05% trypsin/0.02% EDTA, seeded in C6-well plates (Costar Corp., New Bedford, MA), grown to 70% confluency, and used between passages 2 and 3.

Antisense Gene Targeting.

Quiescent EC monolayers were incubated with 50 ng/ml of recombinant VEGF (Collaborative Biomedical Products, Bedford, MA) for 24 h at 37°C in M199 plus 0.1% FCS. At the end of the incubation, EC were washed, harvested by trypsin/EDTA, and lysed in 0.5% Triton X-100, 0.5% NP-40, 0.05 M Tris-Hcl, 0.15 M NaCl plus protease inhibitors. Protein-normalized aliquots of cell extracts were electrophoresed on SDS polyacrylamide gradient gels, transferred to nylon membranes (Millipore Corp.) for 1 h at 1 A, and immunoblotted with 2 μg/ml of a rabbit antibody to survivin or a mouse monoclonal antibody to bcl-2 (Transduction Laboratories, CA) followed by chemiluminescence (Amersham, Arlington Heights, IL) and autoradiography. Samples were sequentially analyzed by Western blotting with a mouse antibody to β-actin to confirm equivalent protein loading. To determine the contribution of survivin to EC protection mediated by VEGF, 2'-O methoxyethyl chimeric phosphorothioate oligonucleotides were utilized. A survivin antisense oligonucleotide with the sequence 5'-TGTGCTATTCTGTGAATT-3' (SEQ ID NO: 1) was characterized previously for its ability to suppress endogenous survivin mRNA expression in T24 bladder and HeLa epithelial carcinoma cells (Li *et al.*, 1999b). A scrambled oligonucleotide with the sequence 5'TAAGCTGTTCTATGTGTT-3' (SEQ ID NO: 2) was used as a control, and also characterized in previous cell culture assays (Li *et al.*, 1999b). Oligonucleotides were synthesized with uniform phosphorothioate linkages, and underlined nucleosides correspond to 2'-O-methoxyethyl nucleosides. Antisense oligonucleotides to platelet-endothelial cell adhesion molecule-1 (PECAM-1, CD31), lymphocyte function-associated molecule-3 (LFA-3, CD58) and intercellular adhesion molecule-1 (ICAM-1, CD54) were synthesized as described above and characterized in previous studies (Baker *et al.*, 1997). For transfection experiments, increasing concentrations of control scrambled or the various antisense oligonucleotide (50-500 nM) were mixed with 1 ml of OPTI-MEM and 6 μl Lipofectin according to manufacturer instructions (Life Technologies, MD), and incubated with serum-starved EC for 8 h. The transfection medium was replaced with M199 plus 0.1% FCS for an additional 18 h followed by

VEGF stimulation for 24 h. Transfection efficiency was monitored by fluorescence microscopy using FITC-conjugated oligonucleotides and was always >85%. To determine the effect of antisense targeting on survivin mRNA expression in proliferating endothelium, EC were transfected with control or the survivin antisense oligonucleotide, harvested after a 24 h culture at 37^{0}C and total RNA was extracted using the Quiagen Rneasy reagent, according to the manufacturer's recommendations. Samples were separated on 1% agarose-formaldehyde gels, transferred to Hybond nylon membranes and hybridized with a ^{32}P-random primed labeled survivin cDNA with visualization of radioactive bands by autoradiography. Northern blots were re-probed with random primed ^{32}P-labeled human G3PDH cDNA to confirm equal loading of the various RNA samples.

Determination of EC Apoptosis.

EC were transfected with increasing concentrations of control or the various antisense oligonucleotides, stimulated with 50 ng/ml VEGF for 16 h at 37^{0}C, and incubated in the presence of 25 μM C-6 ceramide or the combination of TNFα (10 ng/ml Endogen, Woburn, MA) plus cycloheximide (10 μg/ml, Sigma) for an additional 12 h at 37^{0}C. At the end of the incubation, EC (floaters plus attached cells) were harvested, fixed in 70% ethanol, stained with 10 μg/ml propidium iodide plus 100 μg/ml RNase A and 0.05% Triton X-100 in phosphate-buffered saline, pH 7.4, and analyzed for DNA content by flow cytometry, as described (O'Connor *et al.*, 2000). In other experiments, transfected EC stimulated with VEGF and incubated with C-6 ceramide for 12 h at 37^{0}C were harvested, washed in PBS, pH 7.4, and fixed in 4% paraformaldehyde containing 0.25% Triton X-100 for 10 min at 22^{0}C. Cell nuclei were stained with 6.5 μg/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma), 16% polyvinyl alcohol (Air Products and Chemicals, Allentown, PA), and 40% glycerol. Cells were independently scored for morphologic signs of apoptosis (chromatin condensation, DNA fragmentation) using a Zeiss fluorescent microscope.

Caspase Activation.

Transfected EC, stimulated with 50 ng/ml VEGF and incubated with 25 μg/ml C-6 ceramide as described above, were harvested, and solubilized cell extracts were assayed for caspase-3-dependent hydrolysis of the fluorogenic substrate Ac-DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-aldehyde, Pharmingen, San Diego). Fluorescence emissions were quantitated on a spectrofluorometer with excitation wavelength of 360 nm and emission of 460 nm. For biochemical markers of caspase activation, transfected EC treated with VEGF plus ceramide were lysed in 0.25% Triton X-100, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 20 mM HEPES plus protease inhibitors. Protein-normalized aliquots of the various cell extracts were separated by SDS gel electrophoresis, transferred to nylon membranes (Millipore Corp.), and immunoblotted with a 1:5000 dilution of a rabbit antibody to caspase 3 (Transduction Laboratories), or a 1:1000 dilution of a mouse antibody to Poly-ADP ribose polymerase (PARP, Pharmingen, San Diego, CA) followed by chemiluminescence (Amersham, Arlington Heights, IL).

EC migration.

Migration assays were performed using a Boyden chamber (Neuroprobe; Morales-Ruiz *et al.,* 2000). Briefly, quiescent EC were transfected with control or the survivin antisense oligonucleotide, stimulated with VEGF, and detached using 0.05% trypsin and 0.53 mM EDTA. Twenty thousand cells were suspended in M199 medium containing 0.1% BSA and added to the lower chamber. Polycarbonate filters (8-μm diameter) were coated with 100 μg/ml type I collagen. The top half of the chamber was attached and the chamber was incubated in an inverted position at 37°C for 2 h. Increasing concentrations (1-500 ng/ml) of VEGF or D-erythro-sphyngosine-1-phosphate (SPP-1, Calbiochem) were separately added to the upper chamber and incubated for an additional 5 h at 37°C. At the end of the incubation, cells were fixed in 70% ethanol and non-migrating EC on the upper surface of the filter were removed. Migrated cells were stained with Giemsa and counted at 400x magnification in 3 random fields per well (Morales-Ruiz *et al.*, 2000). Each experiment was performed in triplicate

and migration was expressed as the number of total cells counted per well.

Three Dimensional Capillary Formation.

Monolayers of quiescent EC (80% confluency) in C6-well plates were transfected with 500 nM of control or the survivin antisense oligonucleotide. After an 8-h incubation, the transfection medium was replaced with M199 medium containing 0.1% FCS for an additional 18 h at 37°C. Rat-tail type I collagen (3 mg/ml, Becton Dickinson Bedford, MA) in 1/10 volume of 10x DMEM was neutralized with sterile 1 M NaOH and kept on ice. Suspended EC were added to the collagen suspension to a final concentration of $1x10^6$ cells/ml collagen. Ten drops (0.1 ml each) of the EC-collagen mixture were added to a 35-mm plate. Plates were placed in a humidified incubator at 37°C, and the EC-collagen mixtures were allowed to gel for 10 min, after which 3 ml of M199 medium containing 20% FCS, 50 μg/ml ECGS, 100 μg/ml heparin, 100 μg/ml penicillin, and 100 μg/ml streptomycin were added to each plate. EC were allowed to form capillary-like vascular tubes over a 24-h incubation in the presence of 16 nM phorbol myristate acetate (PMA, Sigma). After additional 24 h incubation, EC were washed three times in phosphate buffered saline (PBS), pH 7.2, and supplemented with fresh M199 growth medium in the presence or in the absence of 50 ng/ml VEGF. The cultures were examined by phase-contrast microscopy for the presence of capillary-like vascular tubes during additional 48 h incubation at 37°C as described (Papapetropoulos *et al.*, 1999).

Example 4.1

Inhibition of VEGF-Induced Survivin Expression in EC by Antisense Targeting.

Previous studies demonstrated that a survivin 2'-O methoxyethyl chimeric phosphorothioate antisense oligonucleotide (5'-TGTGCTATTCTGTGAATT-3'; SEQ ID NO: 1) inhibited survivin mRNA and protein expression in HeLa and T24 cancer cell lines (Li *et al.*, 1999b). Consistent with these observations, increasing concentrations of the survivin antisense oligonucleotide suppressed survivin mRNA expression in proliferating EC in a dose-dependent manner, by Northern blotting (Figure 9A). In

contrast, comparable concentrations of a control scrambled oligonucleotide (5'-TAAGCTGTTCTATGTGTT-3'; SEQ ID NO: 2), did not decrease survivin mRNA levels in EC (Figure 9A). In parallel experiments, VEGF stimulation resulted in a ~4-fold increased survivin expression in quiescent endothelium (Figure 9B), and in agreement with previous observations (O'Connor *et al.*, 2000; Tran *et al.*, 1999; Papapetropoulos *et al.*, 2000). Pre-treatment of EC with increasing concentrations of the survivin antisense, but not control oligonucleotide, suppressed VEGF-induction of survivin in a dose-dependent manner by Western blotting (Figure 9B). In contrast, transfection with control or the survivin antisense oligonucleotide did not reduce anti-apoptotic bcl-2 expression in endothelium, by Western blotting (Gerber *et al.*, 1998; Nor *et al.*, 1999) (Figure 9C).

Example 4.2

Antisense Targeting of Survivin Suppresses the Anti-Apoptotic Function of VEGF in EC.

Exposure of quiescent EC to C-6 ceramide resulted in induction of apoptosis as determined by chromatin condensation and DNA fragmentation, by DAPI nuclear staining (Figure 10A, B). Addition of VEGF attenuated ceramide-induced EC apoptosis and restored normal nuclear morphology (Figure 10A, B). Under these experimental conditions, transfection of EC with the survivin antisense oligonucleotide completely reversed the anti-apoptotic function of VEGF against ceramide-induced apoptosis, whereas the control oligonucleotide was ineffective (Figure 10A, B). Similarly, treatment with ceramide or the combination of TNFα plus cycloheximide resulted in a ~7-fold increase in EC apoptosis, as determined by appearance of a cell fraction with hypodiploid, *i.e.* sub-G1, DNA content, by propidium iodide staining and flow cytometry (Figure 10C, D). Addition of VEGF counteracted both ceramide- and TNFα -induced apoptosis in EC by ~45% (Figure 10C, D). However, and consistent with the data presented above, EC transfection with the survivin antisense, but not the control oligonucleotide, suppressed VEGF protection against both cell death-inducing stimuli and restored EC apoptosis to levels observed in the absence of VEGF (Figure 10C, D). Next, it was determined whether suppression of VEGF cytoprotection by survivin

targeting was associated with biochemical hallmarks of apoptosis in EC. Treatment of quiescent EC with ceramide resulted in increased caspase-3 catalytic activity, as determined by hydrolysis of the fluorogenic substrate DEVD-AMC, and in a reaction entirely suppressed by the caspase-3 inhibitor, DEVD-CHO (Figure 11A). This was associated with proteolytic cleavage of ~32 kDa proform caspase-3 and generation of active subunits of ~19 and ~17 kDa (Figure 11B) (Salvesen *et al.*, 1997), and cleavage of the ~115 kDa caspase substrate poly ADP ribose polymerase (PARP) to an ~85 kDa apoptotic fragment (Figure 11C). Under these experimental conditions, addition of VEGF reduced ceramide-induced caspase-3 activity, nearly completely inhibited the generation of ~17 kDa active caspase-3 subunit, and of ~85 kDa PARP fragment (Figure 11A-C). In contrast, EC transfection with the survivin antisense, but not control oligonucleotide, restored the proteolytic generation of ~17 kD active caspase-3 and apoptotic fragmentation of PARP (Figure 11A-C).

Example 4.3

Specificity of Antisense Targeting of Survivin.

It was next determined whether antisense survivin targeting exclusively affected EC viability during VEGF stimulation. Incubation of EC in 0% serum for 24 h resulted in increased caspase-3 activity by DEVD hydrolysis as compared with continuously growing cultures (Figure 12A), and appearance of an apoptotic cell fraction with hypodiploid DNA content by propidium iodide staining and flow cytometry (Figure 12B). Addition of ceramide to these cells further increased caspase-3 activity and generation of EC with hypodiploid DNA content (Figure 12A, B). However, in the absence of VEGF, transfection of EC with control or survivin antisense oligonucleotide did not further enhance caspase-3 activity or generation of apoptotic cells in the presence or in the absence of ceramide (Figure 12A, B).

In other experiments, EC transfection with antisense oligonucleotides to PECAM-1(CD31), LFA-3 (CD58), or ICAM-1 (CD54) resulted in concentration-dependent suppression of the various targeted mRNAs, by Northern blotting, as described

previously (Baker *et al.*, 1997). However, when analyzed for nuclear morphology by DAPI staining, expression of the various antisense oligonucleotides did not significantly reduce the anti-apoptotic function of VEGF against ceramide-induced EC death (Figure 13). In contrast, and in agreement with the data presented above, EC transfection with the survivin antisense, but not control oligonucleotide blocked the cytoprotective effect of VEGF in ceramide-treated cultures (Figure 13).

Example 4.4

Role of Survivin in VEGF-Induced EC Migration and Remodeling.

The potential role of survivin targeting on other angiogenic responses induced by VEGF, *i.e.* EC migration and stabilization of three-dimensional vascular networks (Risau *et al.*, 1997) was next investigated. First, stimulation with VEGF or SPP-1 resulted in EC chemotaxis and migration in a specific and concentration-dependent manner (Figure 15), in agreement with previous observations (Morales-Ruiz *et al.*, 2000). Transfection of VEGF-stimulated EC with inhibitory concentrations of control or the survivin antisense oligonucleotide failed to decrease EC migration in response to VEGF or SPP-1 (Figure 15). In a second series of experiments, the effect of antisense survivin targeting on the non-proliferative, remodeling phase of VEGF-induced angiogenesis was investigated. Addition of VEGF to EC-collagen gels primed with PMA and transfected with control oligonucleotide supported the generation of viable three-dimensional capillary-like structures, which persisted throughout a 3-day culture at 37°C (Figure 15) (Ilan *et al.* 1998). In contrast, no viable capillaries were formed in the absence of PMA (not shown), and withdrawal of VEGF resulted in rapid involution of three-dimensional vascular networks over a 3-day culture (Figure 15), in agreement with previous observations (Ilan *et al.*, 1998). Under these experimental conditions, transfection of EC with the survivin antisense oligonucleotide completely reversed the protective effect of VEGF on capillary formation and maintenance and resulted in complete involution of three dimensional vascular networks during a 3-day culture (Figure 15).

Although the present invention has been described in detail with reference to

examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents, patent applications and publications referred to in this application are herein incorporated by reference in their entirety.

REFERENCES

The following references are herein incorporated by reference in their entirety:

Ackermann *et al.*, J Biol Chem 1999, 274:11245-11252

Adams *et al.*, Science 1998, 281:1322-1326

Alon *et al.*, Nat Med 1995, 1:1024-1028

Altieri *et al.*, Lab Invest 1999, 79:1327-1333

Ambrosini *et al.*, Nat Med 1997, 3:917-921

Bach *et al.*, Immunol Today 1997, 18:483-486

Baker *et al.*, J Biol Chem 1997, 272:11994-12000

Benjamin *et al.*, Proc Natl Acad Sci U S A 1997, 94:8761- 8766

Bjorkerud S *et al.*, Am J Pathol 1996, 149:367-380

Blaese *et al.*, Science 1995, 270:475-480

Brooks *et al.*, Cell 1998, 92:391-400

Carmeliet , Nat Med 2000, 6:389-395

Chen *et al.*, J Neurosci 1998, 18:4914-4928

Chen *et al.*, Neoplasia 2000, 2:236-242

Claesson-Welsh *et al.*, Proc Natl Acad Sci U S A 1998, 95:5579-5583

Davis *et al.*, Cell 1996, 87:1161-9

De Luca *et al.*, J. Biol. Chem. 1994, 269:19193-19196

Deveraux *et al.*, Genes Dev 1999, 13:239-252

Duckett *et al.*, EMBO J 1996, 15:2685-2694

Folkman *et al.*, J. Biol. Chem. 1992, 267 (16), 10931-10934

Fraser *et al.*, Curr Biol 1999, 9:292-301

Frisch *et al.*, Curr Opin Cell Biol 1997, 9:701-6

Fujio *et al.*, J. Biol. Chem. 1999, 274:16349-54

Gerber *et al.*, J. Biol. Chem. 1998a, 273:13313-13316

Gerber *et al.*, J. Biol. Chem. 1998b, 273:30336-30343

Hanahan *et al.*, Cell 1996, 86:353-364

Hanahan, Science 1997, 277: 48-50

Hay *et al.,* Cell 1995, 83:1253-1262

Hengartner, Nature 2000, 407:770-776

Ilan *et al.*, J Cell Sci 1998, 111:3621-3631

Isik *et al.*, J Cell Physiol 1998, 175:149-155

Isner *et al.*, Lancet 1996, 348:370

Karsan *et al.*, J Atheroscler Thromb 1996, 3:75-80

Kobayashi *et al.*, Proc Natl Acad Sci U S A 1999, 96:1457-1462

Koblizek *et al.,* Curr. Biol. 1998, 8:529-532.

Kontos *et al.*, Mol Cell Biol 1998, 18:4131-40.

Ledley *et al.*, J. Pediatrics 1987, 110:1

Ledley *et al.*, Proc. Natl. Acad. Sci. 1987, 84:5335-5339

Li *et al.*, Nature 1998, 396:580-4

Li *et al.,* Biochem. J. 1999a, 344: 305-11

Li *et al.*, Nat Cell Biolog 1999b, 1:461-466

Lin *et al.*, Proc Natl Acad Sci U S A 1998, 95:8829-8834

Lim *et al.,* Proc. Natl. Acad. Sci. USA 1989, 86:8892-8896

Liston, P. *et al.,* Nature 1996, 379:349-353

Maisonpierre *et al.*, Science 1997, 277:55-60

Milligan *et al.*, J. Med. Chem. 1993, 36(14): 1923-1937

Morales-Ruiz *et al.*, Circ Res 2000, 86:892-896

Morgan *et al.*, Science 1987, 237:1476-1479

Moullier *et al.*, Nature Genetics 1993, 4:154

Nicolau *et al.*, Proc. Natl. Acad. Sci. U.S.A. 1983, 80:1068

Nor *et al.*, Am J Pathol 1999, 154:375-384

O'Connor *et al.*, Am J Pathol 2000a, 156:393-398

O'Connor *et al.*, Proc Natl Acad Sci USA 2000b, 97:13103-13107

Olie *et al.*, Cancer Res 2000, 60:2805-2809

Olivetti *et al.,* J Mol Cell Cardiol 1996, 28:2005-2016

Olivetti *et al.,* N Engl J Med 1997, 336:1131-1141

O'Reilly *et al.*, Nat Med 1996, 2:689-92

Papapetropoulos *et al.*, Lab Invest 1999, 79:213-23.

Papapetropoulos *et al.*, J Biol Chem 2000, 275:9102-9105

Raiasubramanian *et al.*, ASAIO Journal 1994, 40(3): M584-9

Reed, J Clin Oncol 1999, 17:2941-2953

Risau, Nature 1997, 386:671-674

Rosenberg *et al.*, Science 1988, 242:1575-1578

Rothe, M. *et al.*, Cell 1995, 83:1243-1252

Roy *et al.*, Cell 1995, 80:167-178

Roy *et al.*, Blood 1997, 595:2645

Rudin *et al.*, Annu Rev Med 1997, 48:267-281

Ruegg *et al.*, Nat Med 1998, 4:408-414

Salvesen *et al.*, Cell 1997, 91:443-446

Sierra-Honigman *et al.*, Science 1998, 281:1683-1686

Stehlik *et al.*, J Exp Med 1998, 188:211-216

Stromblad *et al.*, Chem Biol 1996, 3:881-885

Takeshita *et al.*, Laboratory Investigation 1996, 75:487-502

Tamm *et al.*, Cancer Res 1998, 58:5315-5320

Thompson, Science 1995, 267:1456-1462

Tran *et al.*, Biochem Biophys Res Commun 1999, 264:781-788

Uhlmann *et al.*, Chemical Reviews 1990, 90:543-584

Uren *et al.*, Proc Natl Acad Sci U S A 1999, 96:10170-10175

Vaux DL *et al.*, Cell 1999, 96:245-254

Velculescu *et al.*, Nat Genet 1999, 23:387-388

Wilson *et al.*, Proc. Natl. Acad. Sci. 1990, 87:8437-8441

Witzenbichler *et al.*, J Biol Chem 1998, 273:18514-21.

Yuan *et al.*, Proc Natl Acad Sci U S A 1996, 93:14765-14770

Zhao *et al.*, J Cell Sci 2000, 113:4363-4371

WHAT IS CLAIMED:

1. A method of promoting angiogenesis comprising the step of providing to a cell or tissue an apoptosis inhibiting concentration of survivin or survivin activity.

2. A method of claim 1, wherein the survivin concentration or activity is increased by providing an agent selected from the group consisting of a survivin polypeptide, a survivin transgene, a survivin peptidomimetic and an agent that modulates the expression of survivin in the cell or tissue.

3. A method according to claim 2, wherein the agent is angiopoietin-1 or VEGF.

4. A method according to claim 2, wherein the agent is activated serine-threonine kinase Akt.

5. A method of either of claims 1 or 2, wherein the agent is provided in an implant.

6. A method of claim 5, wherein the implant is coated or impregnated with a survivin transgene.

7. A method of claim 6, wherein the transgene is operatively linked to an expression control element.

8. A method of claim 7, wherein the transgene is contained within a vector.

9. A method of claim 7, wherein the transgene is contained within a transfection facilitating composition.

10. A method of claim 9, wherein the transfection facilitating composition is a transfection facilitating lipid or a transfection facilitating particle.

11. A method of either of claims 1 or 2, wherein the method treats a condition by inducing compensatory angiogenesis.

12. A method according to claim 11, wherein the condition is an ischemic disease.

13. A method according to claim 12, wherein the ischemic disease is caused by myocardial infarction, peripheral vascular occlusion, brain ischemia or stroke .

14. A method of promoting endothelial cell angiogenesis comprising the step of providing to subject endothelial cells engineered to express an apoptosis inhibiting amount of survivin.

15. A method of claim 14, wherein the cells are provided in an implant.

16. A method according to claim 15, wherein the implant is a stent.

17. A method of claim 15, wherein the implant is coated or impregnated with a survivin transgene.

18. A method of claim 17, wherein the transgene is operatively linked to an expression control element.

19. A method of claim 18, wherein the transgene is contained within a vector.

20. A method of claim 17, wherein the transgene is contained within a transfection facilitating composition.

21. A method of claim 20, wherein the transfection facilitating composition is a transfection facilitating lipid or a transfection facilitating particle.

22. A method of claim 14, wherein the method treats a condition by inducing compensatory angiogenesis.

23. A method according to claim 22, wherein the condition is an ischemic disease.

24. A method according to claim 23, wherein the ischemic disease is caused by myocardial infarction, peripheral vascular occlusion, brain ischemia or stroke .

25. A method of preventing a vasculoproliferative disease comprising the step of providing to a patient an agent which modulates the expression or activity of survivin.

26. A method of claim 25, wherein the agent down-regulates the expression of survivin.

27. A method of claim 26, wherein the agent is a survivin antisense molecule.

28. A method of claim 26, wherein the agent is provided in an implant.

29. A method of claim 28, wherein the implant is a stent.

30. A method of claim 29, wherein the stent is coated or impregnated with a survivin antisense molecule.

31. A method of claim 30, wherein the survivin antisense molecule is contained within a transfection facilitating composition.

32. A method of claim 31, wherein the transfection facilitating composition is a transfection facilitation lipid or a transfection facilitating particle.

33. A method of claim 25, wherein the vasculoproliferative disease is restenosis, vascular bypass graft occlusion or transplant coronary vasculopathy.

34. The method of either of claims 11 or 22, wherein the condition is selected from the group consisting of tissue damage or a wound.

35. A device suitable for implantation into a patient, wherein the device is coated or impregnated with a survivin transgene or survivin antisense molecule.

36. A device suitable for implantation into a patient, wherein the device comprises endothelial cells engineered to express an apoptosis inhibiting amount of survivin.

37. A method of inhibiting angiogenesis in a cell comprising administering an agent that inhibits surivin.

38. The method of claim 37, wherein the cell is tumorigenic.

39. A method of inhibiting VEGF induced angiogenesis comprising administering an agent that inhibits survivin.

40. A method of inhibiting VEGF induced activity comprising administering an agent that inhibits survivin.

41 The method of claim 40, wherein the VEGF induced activity is capillary formation.

42. The method of any one of claims 37, 38, 39, 40, or 41 wherein the agent is an inhibitor of survivin function or an inhibitor of survivin expression.

43. The method of claim 42, wherein the agent is a survivin antibody, a survivin antisense molecule, or an inhibitor of Akt phosphorylation.

44. A method of treating a patient in need thereof with an agent that modulates the expression or function of survivin and an immunomodulatory agent.

45. The method of claim 44, wherein the agent inhibits the expression or function of survivin.

46. The method of claim 44, wherein the agent promotes the expression or function of survivin.

47. The method of claim 44, wherein the agents is an immunosuppressive agent.

48. The method of claim 44, wherein the patient has received a graft.

49. the method of claim 45, wherein the patient has received a transplant.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Yale University

International Application No.:
(based on US 60/172,991)

International Application Filed: December 21, 2000

Assistant Commissioner for Patents
Box PCT
Washington, D.C. 20231

Attn: RO/US - Authorized Officer

Dear Authorized Officer:

STATEMENT ACCOMPANYING SEQUENCE LISTING

The undersigned hereby states upon information and belief that the Sequence Listing submitted concurrently herewith does not include matter which goes beyond the content of the application as filed and that the information recorded on the diskette submitted concurrently herewith is identical to the written Sequence Listing submitted herewith.

Respectfully submitted,

Date: 12.21.00

By: Rosanne Kosson
Name: Rosanne Kosson
Registration No. 46,840

MORGAN, LEWIS & BOCKIUS LLP
1800 M Street, N.W.
Washington, D.C. 20036
Tel: (202) 467-7000
Fax: (202) 467-7258

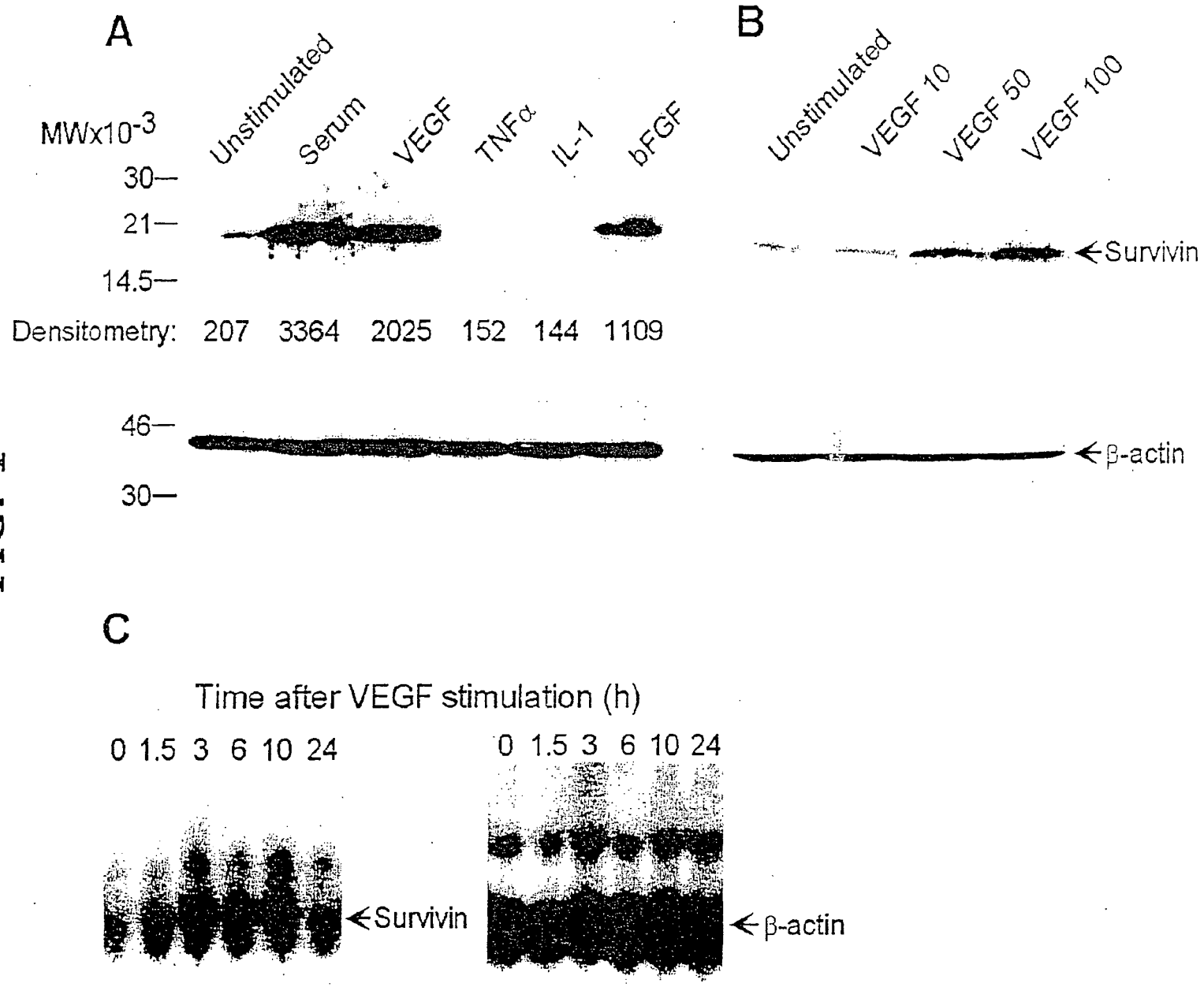
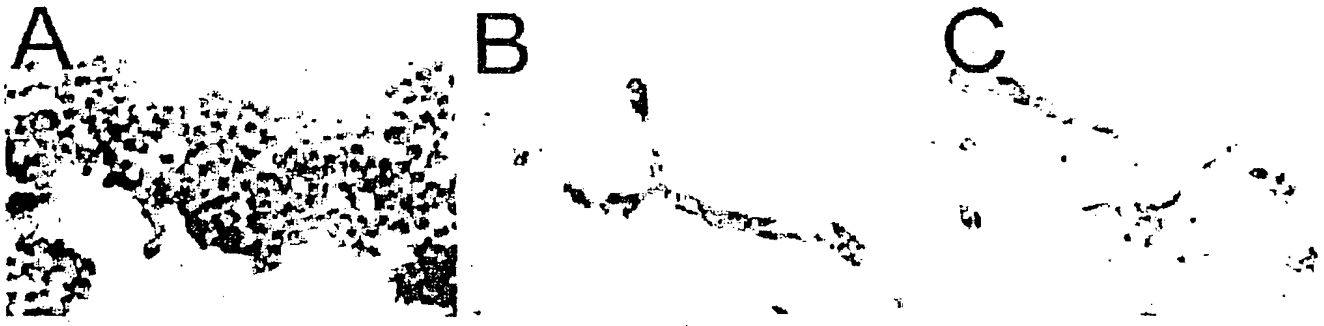

A
Unstimulated
Serum
VEGF
TNFα
IL-1
bFGF
MWx10-3
30—
21—
14.5—
Densitometry: 207 3364 2025 152 144 1109
46—
30—
B
Unstimulated
VEGF 10
VEGF 50
VEGF 100
←Survivin
←β-actin
C
Time after VEGF stimulation (h)
0 1.5 3 6 10 24
0 1.5 3 6 10 24
←Survivin
←β-actin

FIG. 1

FIG. 2



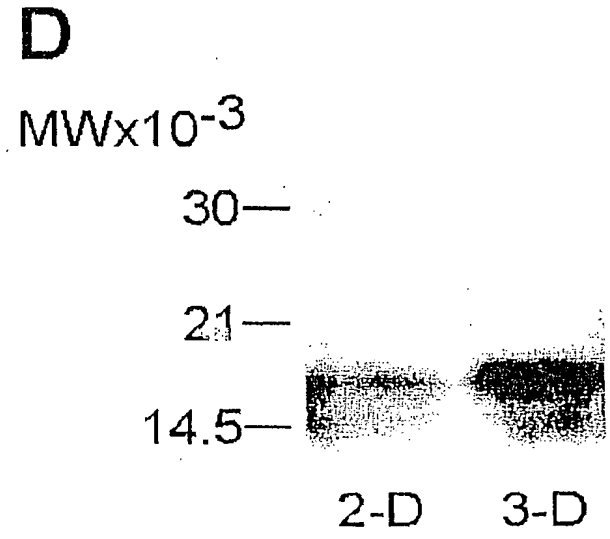
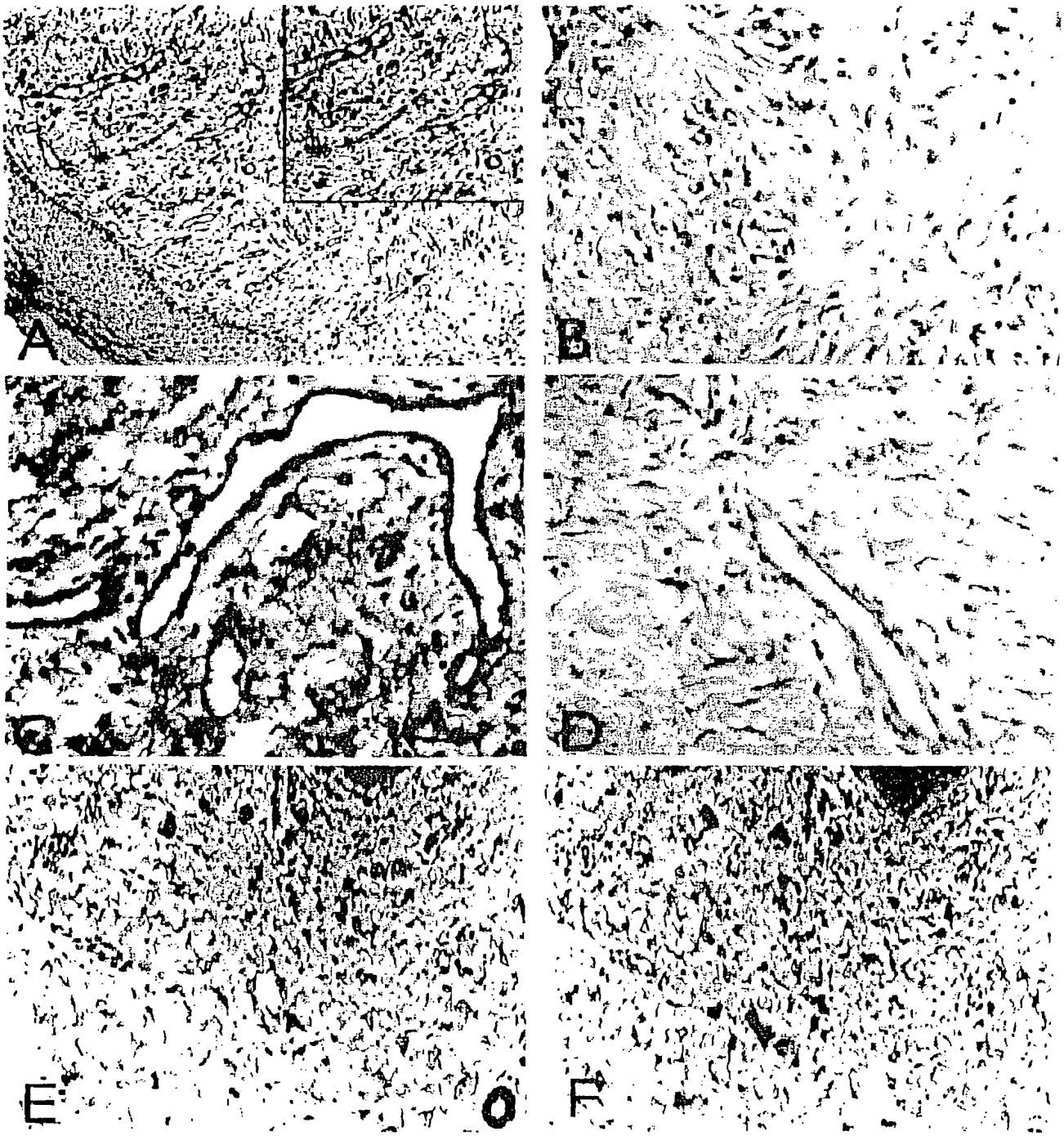


FIG. 3



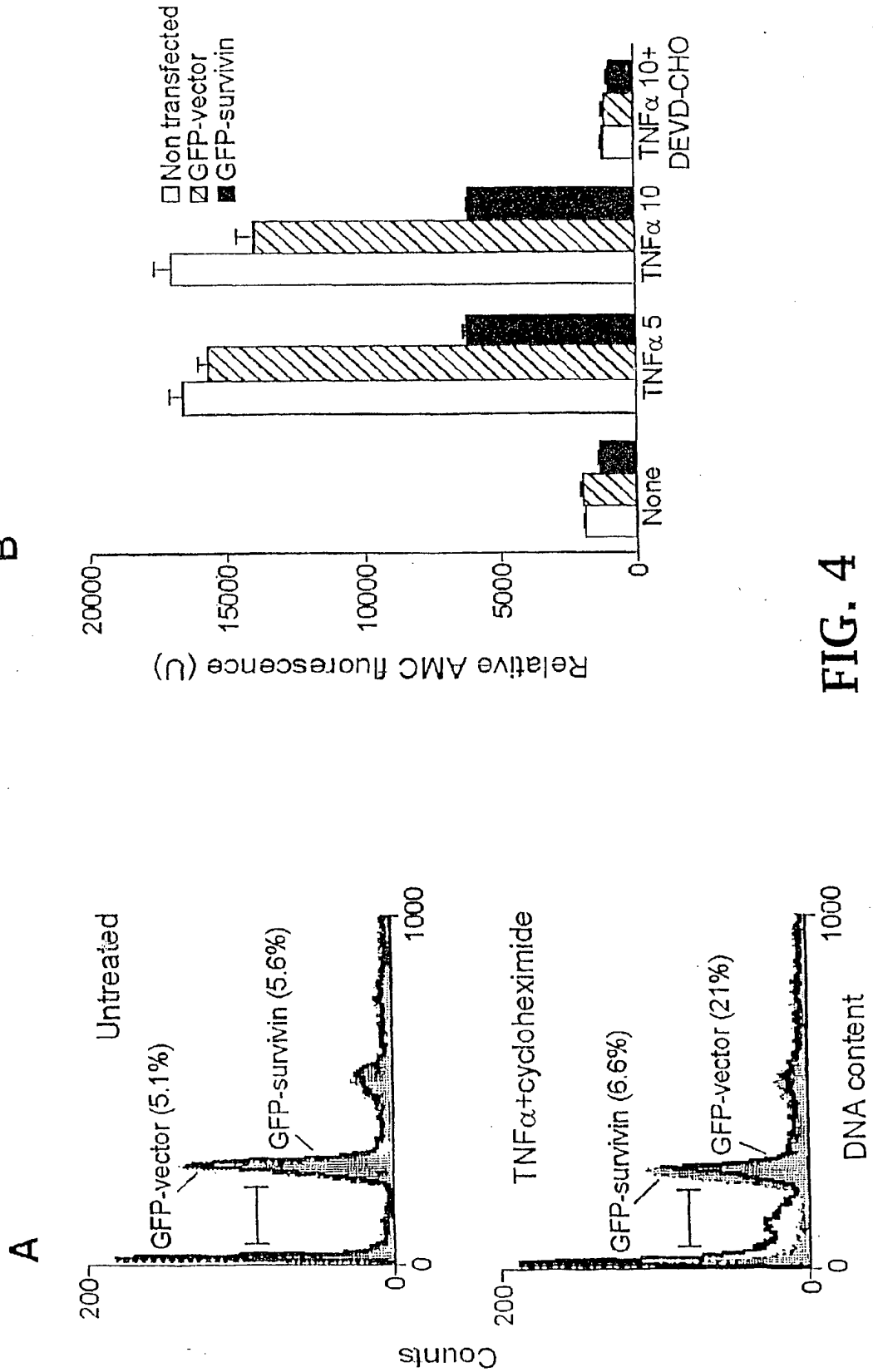

A
Untreated
GFP-vector (5.1%)
GFP-survivin (5.6%)
TNFα+cycloheximide
GFP-survivin (6.6%)
GFP-vector (21%)
Counts
DNA content
200
0
1000
B
Non transfected
GFP-vector
GFP-survivin
Relative AMC fluorescence (U)
20000
15000
10000
5000
0
None
TNFα 5
TNFα 10
TNFα 10+ DEVD-CHO

FIG. 4

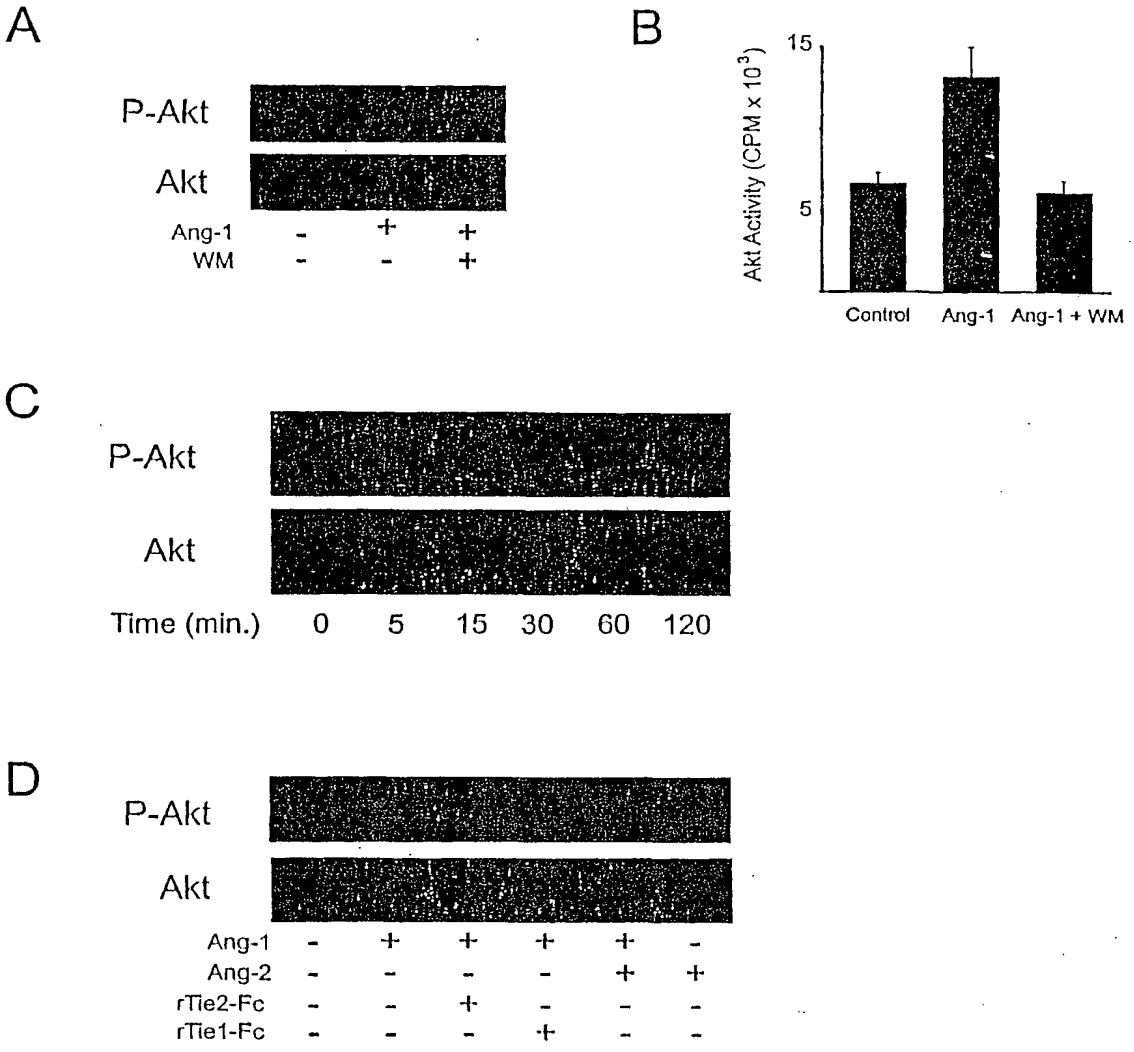

A
P-Akt
Akt
Ang-1 - + +
WM - - +
B
Akt Activity (CPM x 10³)
15
5
Control
Ang-1
Ang-1 + WM
C
P-Akt
Akt
Time (min.) 0 5 15 30 60 120
D
P-Akt
Akt
Ang-1 - + + + + -
Ang-2 - - - - + +
rTie2-Fc - - + - - -
rTie1-Fc - - - + - -

FIG. 5

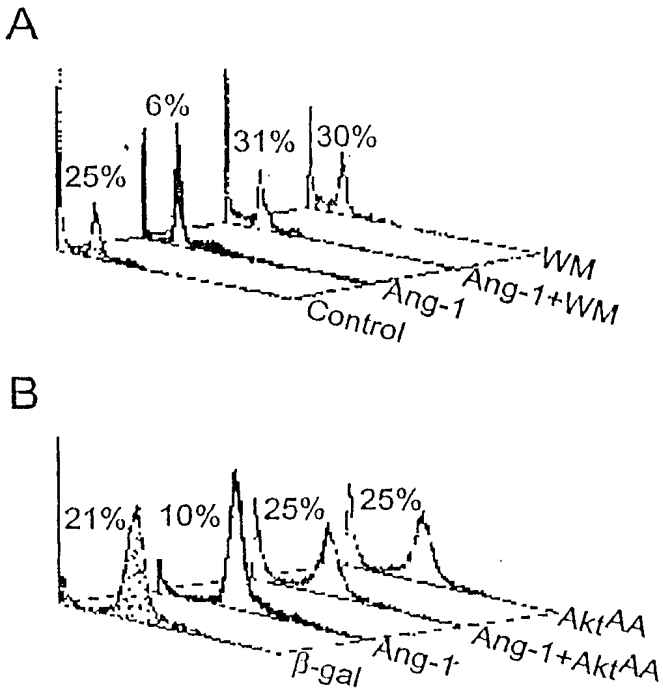

A
6%
31%
30%
25%
WM
Ang-1+WM
Ang-1
Control
B
21%
10%
25%
25%
AktAA
Ang-1+AktAA
Ang-1
β-gal
C
α-P-Akt
α-Akt
WM - - + +
Ang-1 - + - +

FIG. 6

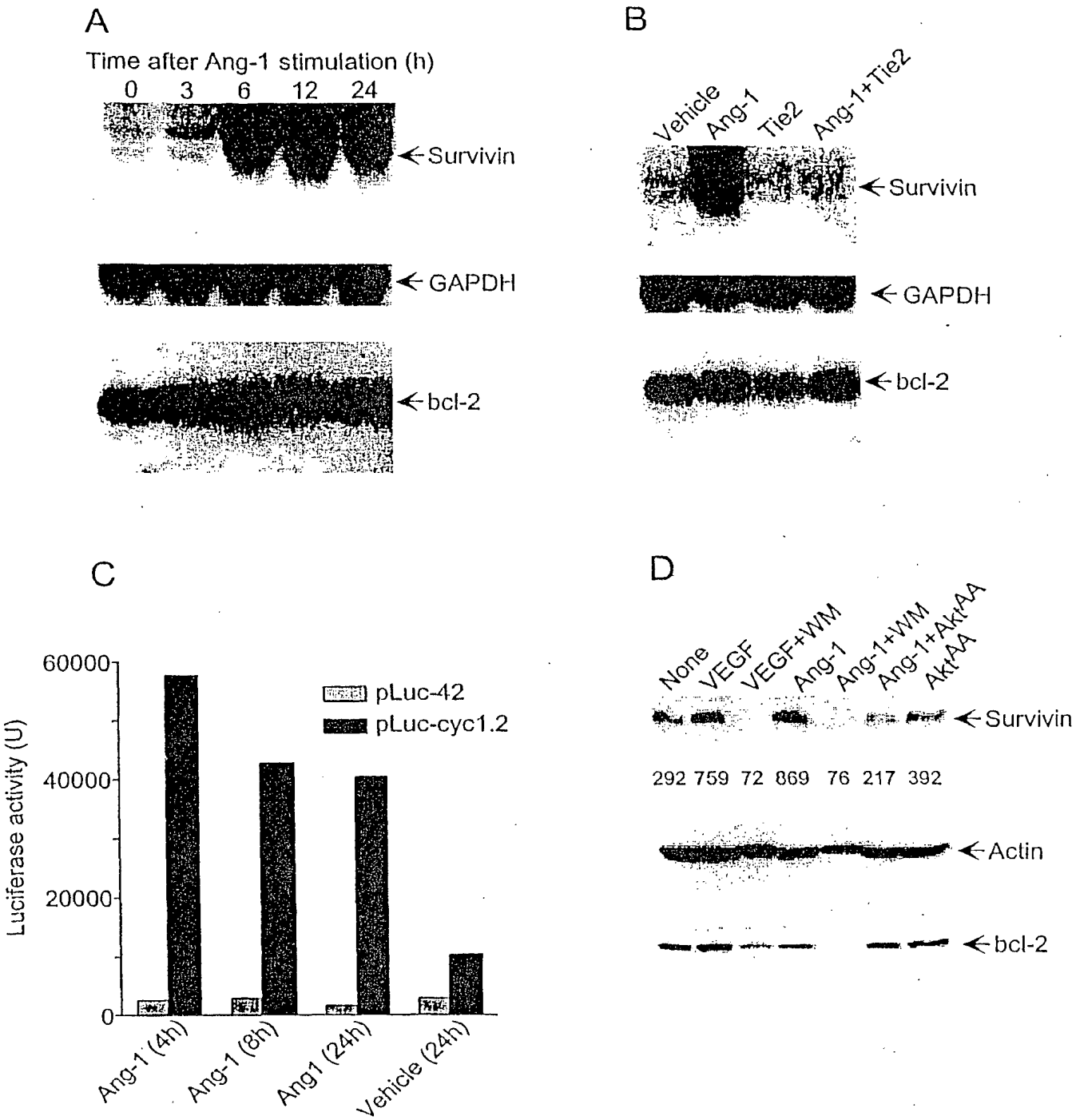

A
Time after Ang-1 stimulation (h)
0 3 6 12 24
Survivin
GAPDH
bcl-2
B
Vehicle
Ang-1
Tie2
Ang-1+Tie2
Survivin
GAPDH
bcl-2
C
Luciferase activity (U)
60000
40000
20000
0
pLuc-42
pLuc-cyc1.2
Ang-1 (4h)
Ang-1 (8h)
Ang1 (24h)
Vehicle (24h)
D
None
VEGF
VEGF+WM
Ang-1
Ang-1+WM
Ang-1+AktAA
AktAA
Survivin
292 759 72 869 76 217 392
Actin
bcl-2

FIG. 7

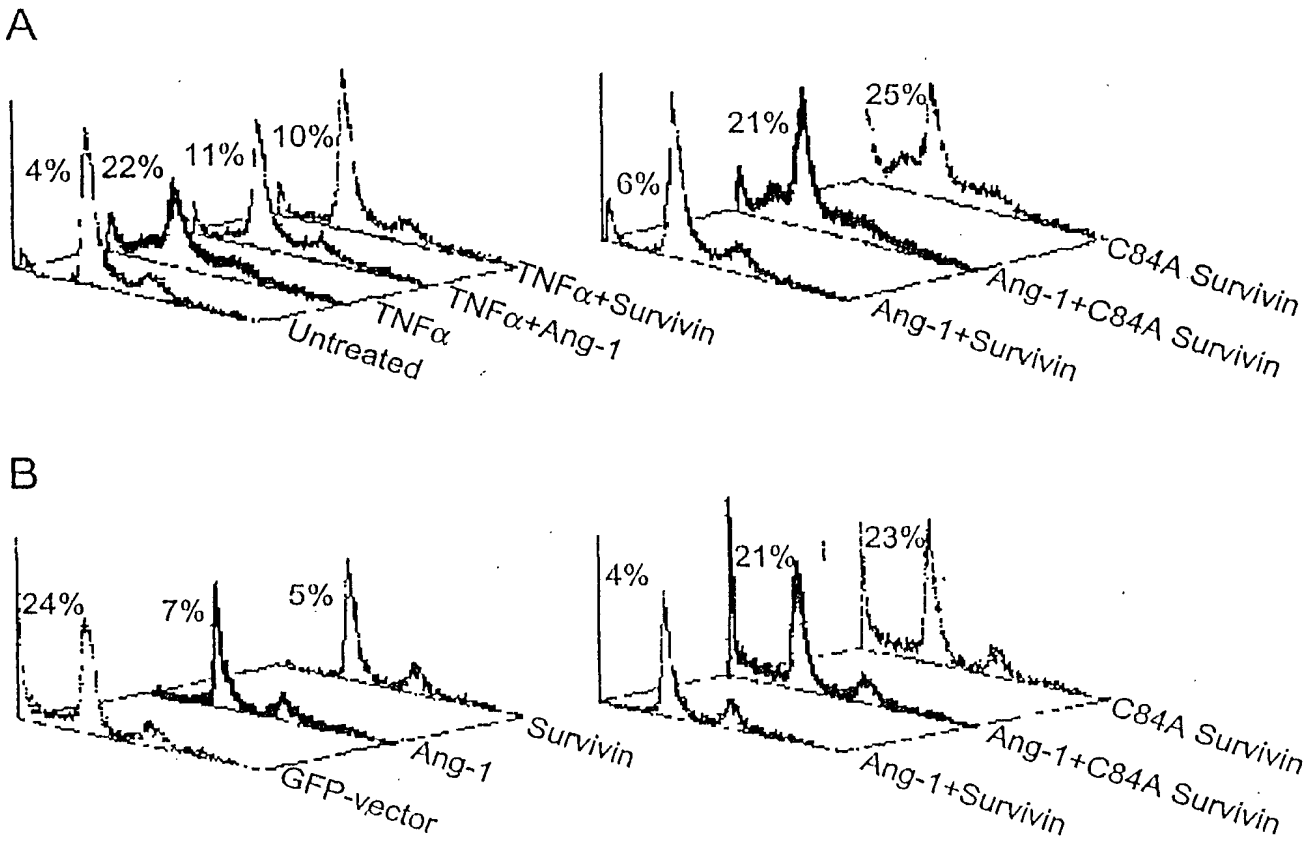

A
4%
22%
11%
10%
Untreated
TNFα
TNFα+Ang-1
TNFα+Survivin
6%
21%
25%
Ang-1+Survivin
Ang-1+C84A Survivin
C84A Survivin
B
24%
7%
5%
GFP-vector
Ang-1
Survivin
4%
21%
23%
Ang-1+Survivin
Ang-1+C84A Survivin
C84A Survivin

FIG. 8

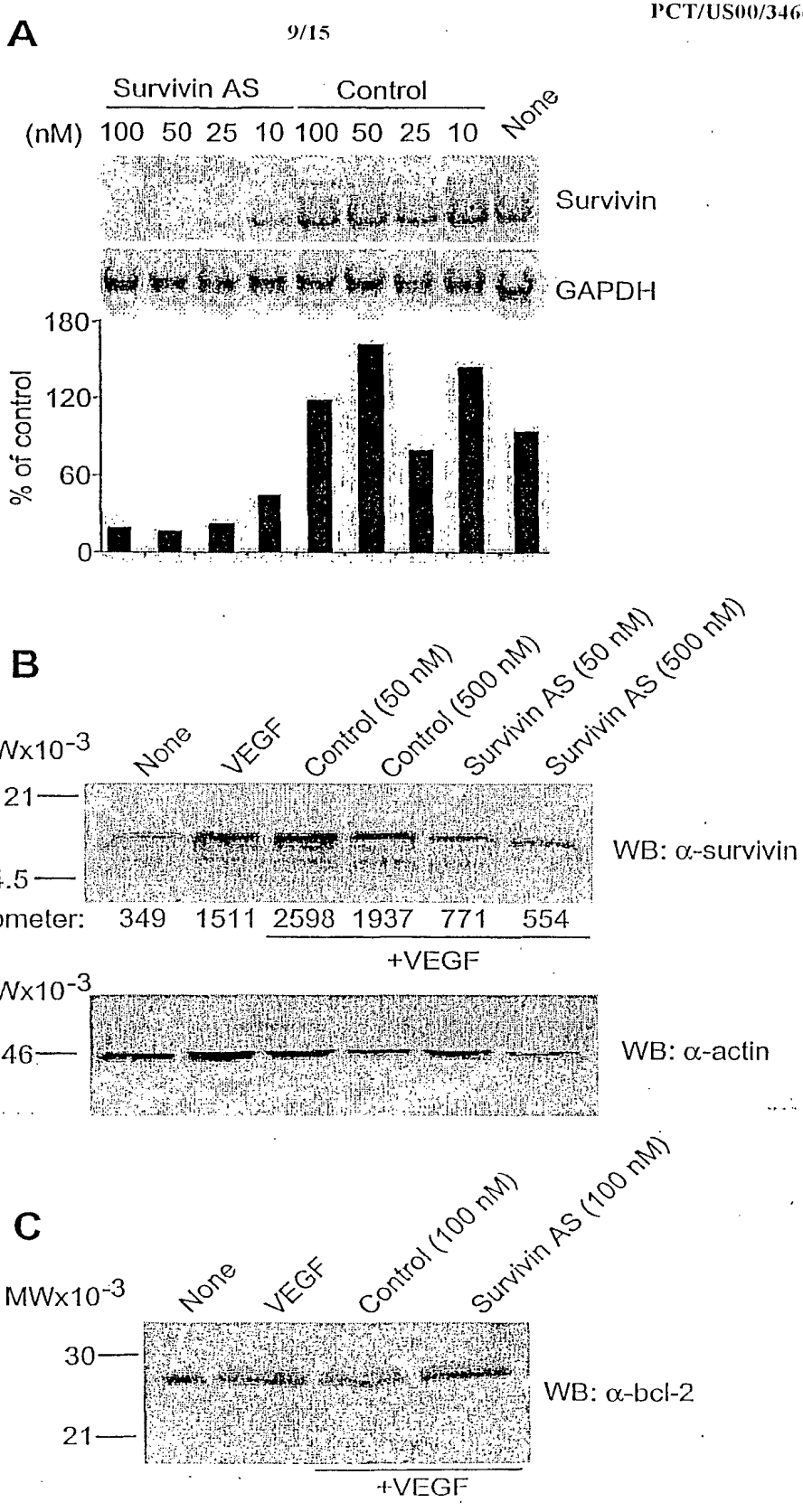

A
Survivin AS
Control
(nM) 100 50 25 10 100 50 25 10 None
Survivin
GAPDH
180
120
60
0
% of control
B
MWx10-3
None
VEGF
Control (50 nM)
Control (500 nM)
Survivin AS (50 nM)
Survivin AS (500 nM)
21
14.5
WB: α-survivin
Densitometer: 349 1511 2598 1937 771 554
+VEGF
MWx10-3
46
WB: α-actin
C
MWx10-3
None
VEGF
Control (100 nM)
Survivin AS (100 nM)
30
21
WB: α-bcl-2
+VEGF

Figure 9

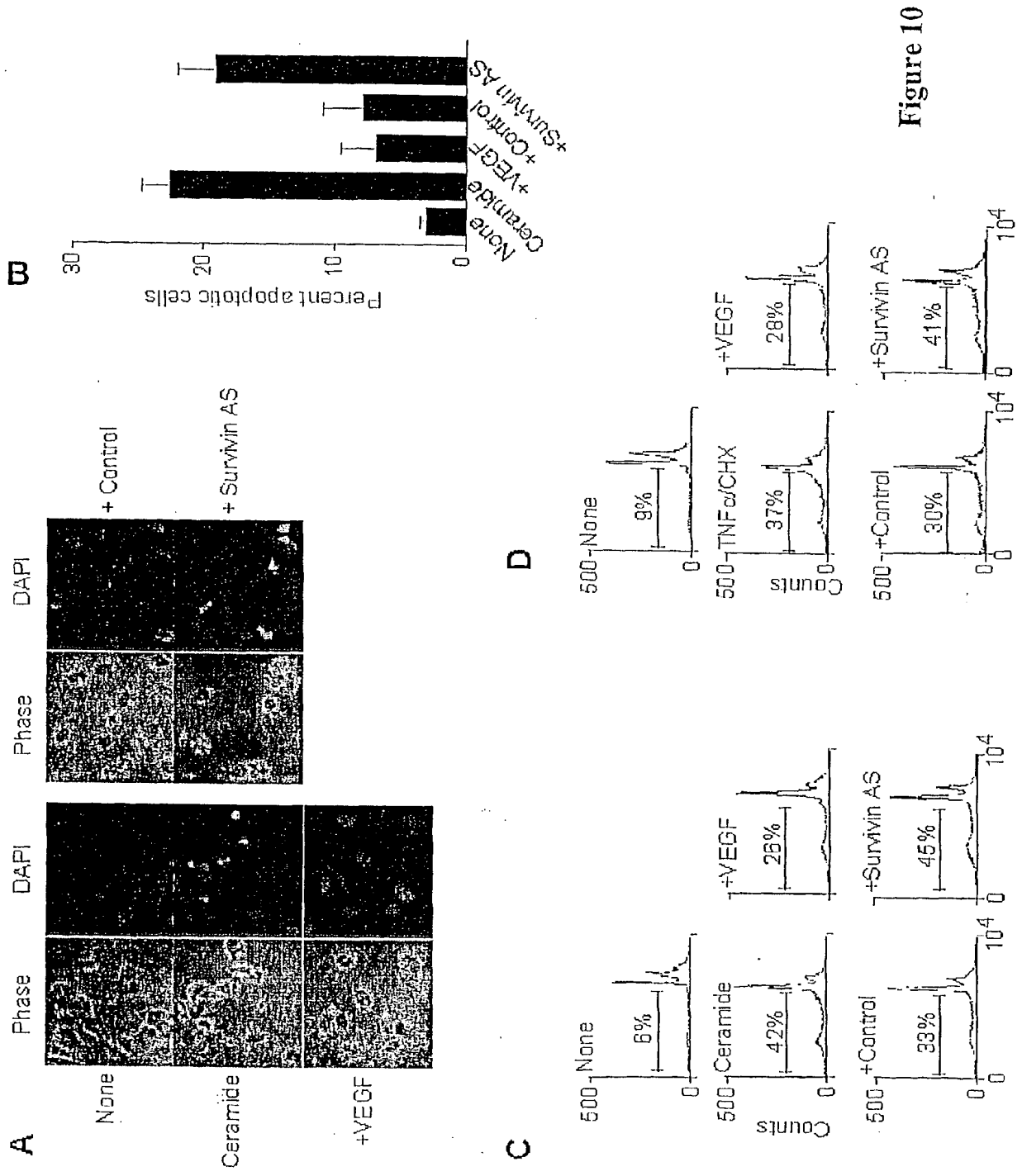

A
Phase
DAPI
Phase
DAPI
+ Control
+ Survivin AS
None
Ceramide
+VEGF
B
Percent apoptotic cells
30
20
10
0
None
Ceramide
+VEGF
+Control
+Survivin AS
C
500
None
6%
Ceramide
42%
+VEGF
26%
+Control
33%
+Survivin AS
45%
Counts
0
10^4
D
500
None
9%
TNFα/CHX
37%
+VEGF
28%
+Control
30%
+Survivin AS
41%
Counts
0
10^4

Figure 10

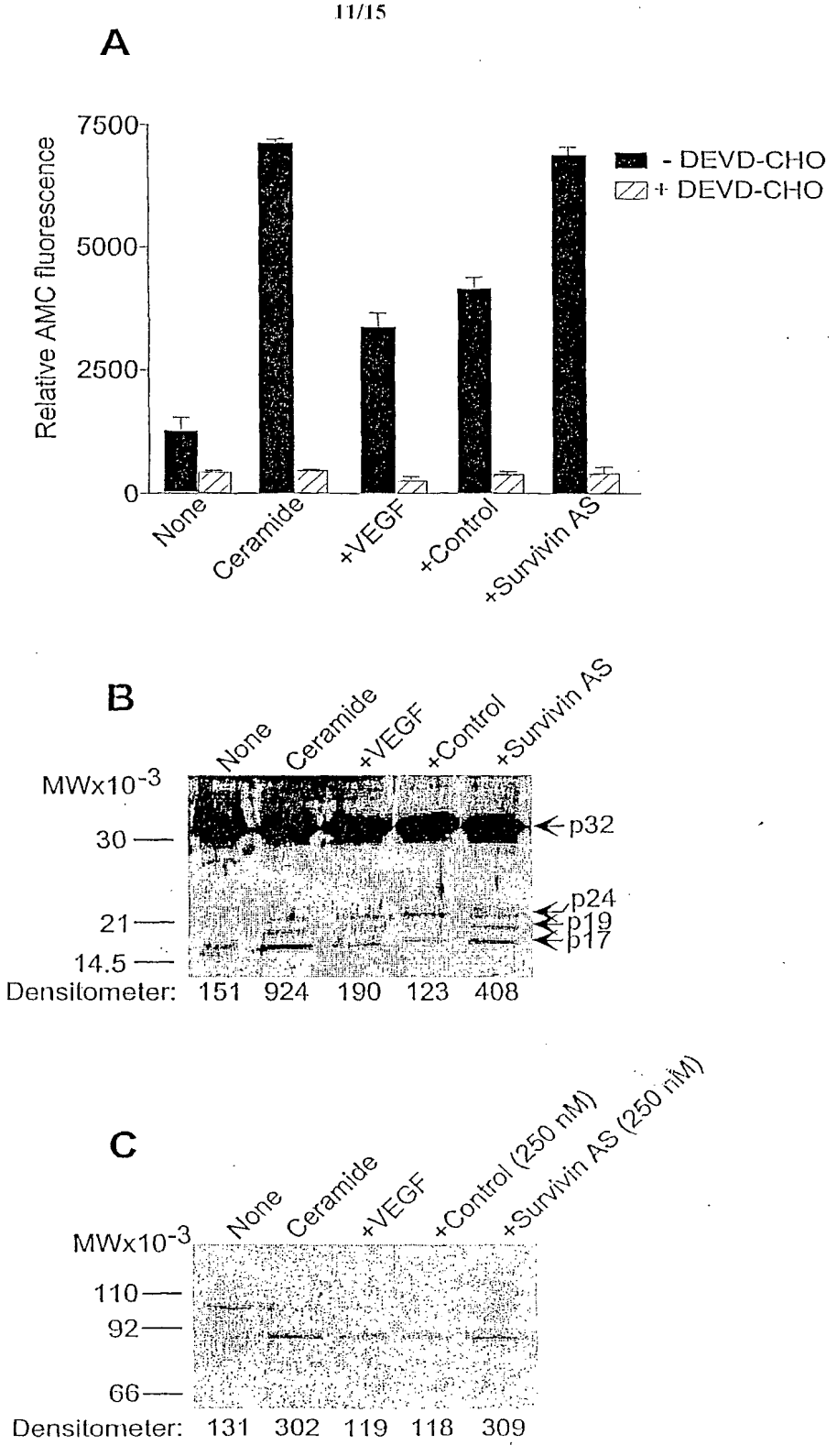

A
Relative AMC fluorescence
7500
5000
2500
0
- DEVD-CHO
+ DEVD-CHO
None
Ceramide
+VEGF
+Control
+Survivin AS
B
None
Ceramide
+VEGF
+Control
+Survivin AS
MWx10-3
30
21
14.5
p32
p24
p19
p17
Densitometer: 151 924 190 123 408
C
None
Ceramide
+VEGF
+Control (250 nM)
+Survivin AS (250 nM)
MWx10-3
110
92
66
Densitometer: 131 302 119 118 309

Figure 11

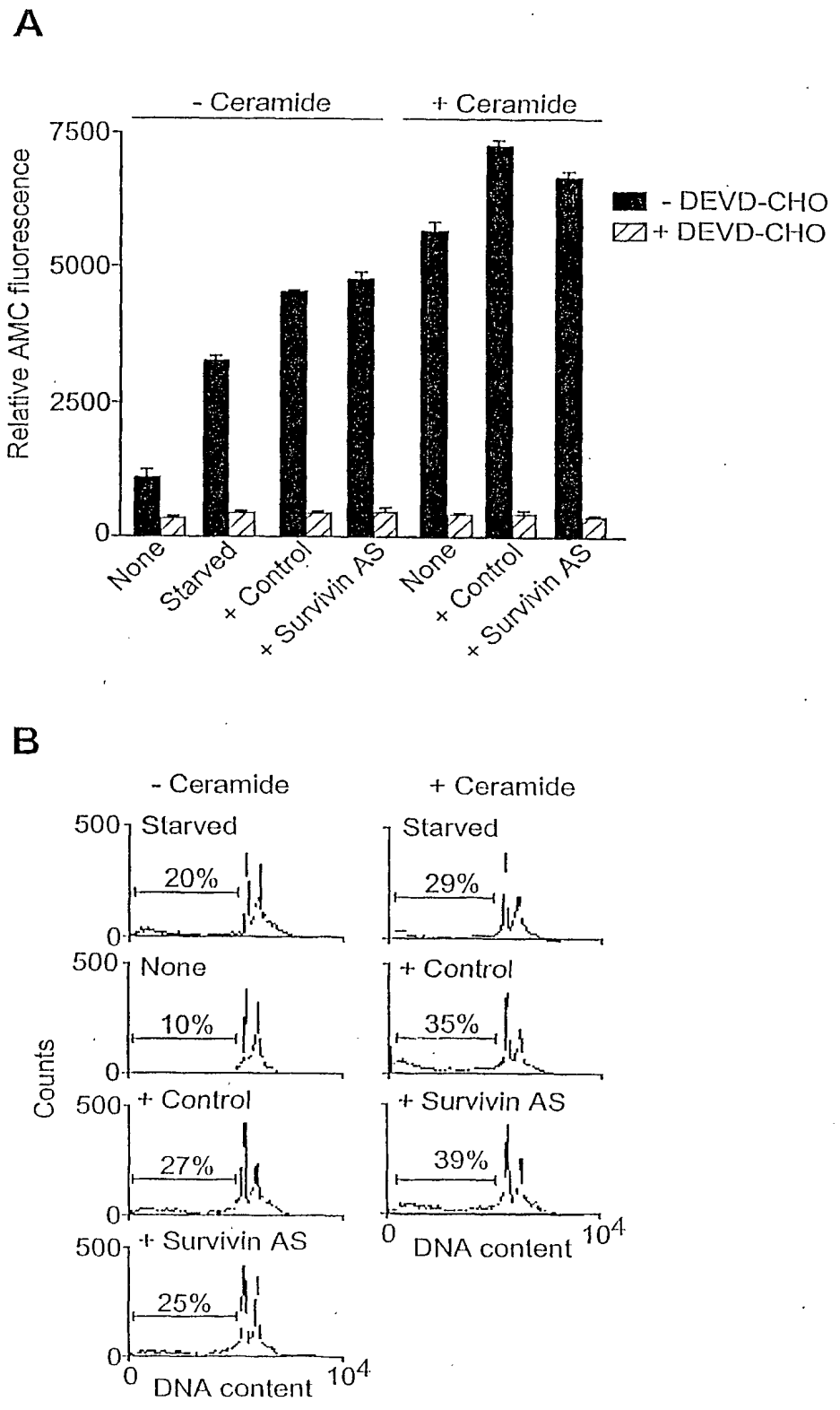

A
- Ceramide
+ Ceramide
7500
5000
2500
0
Relative AMC fluorescence
- DEVD-CHO
+ DEVD-CHO
None
Starved
+ Control
+ Survivin AS
None
+ Control
+ Survivin AS
B
- Ceramide
+ Ceramide
Starved
20%
Starved
29%
None
10%
+ Control
35%
+ Control
27%
+ Survivin AS
39%
+ Survivin AS
25%
500
0
Counts
0
DNA content
10^4

Figure 12

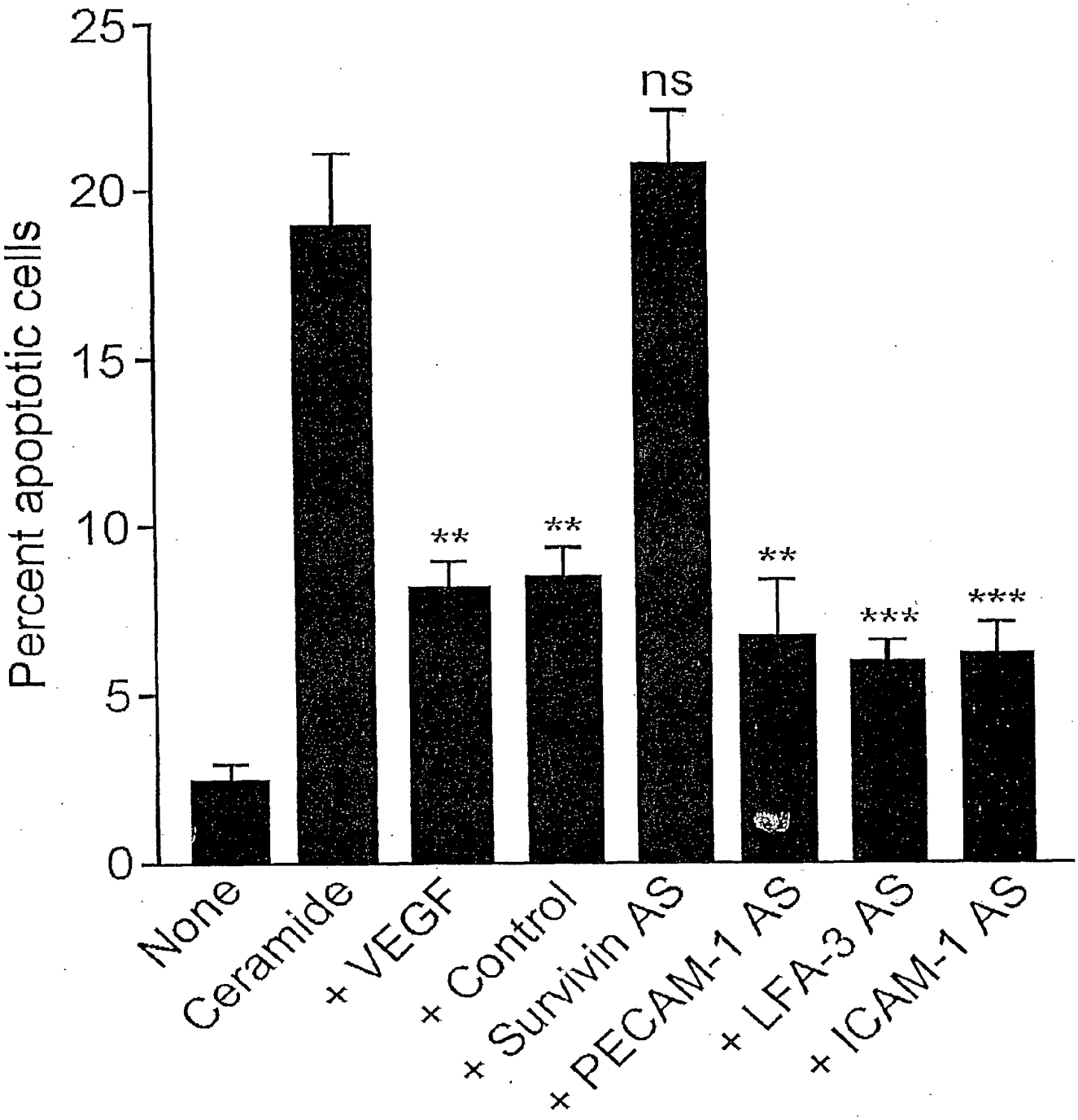
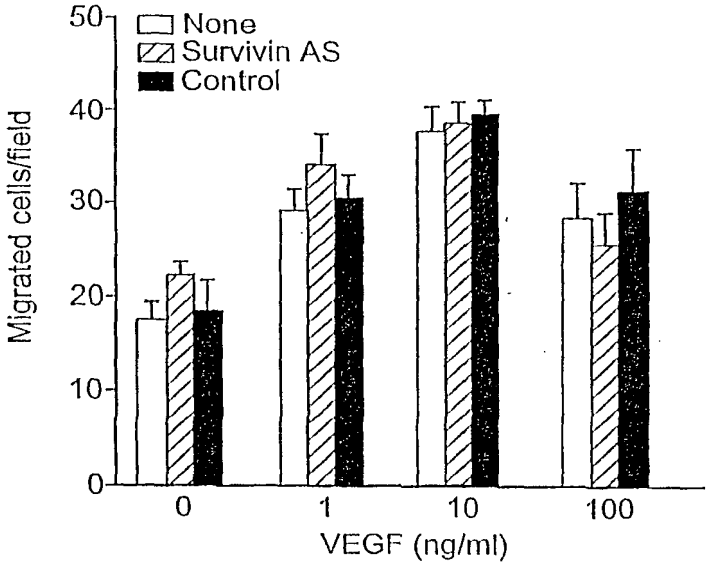

Percent apoptotic cells
25
20
15
10
5
0
ns
**
**
**
***
***
None
Ceramide
+ VEGF
+ Control
+ Survivin AS
+ PECAM-1 AS
+ LFA-3 AS
+ ICAM-1 AS

Figure 13


50
40
30
20
10
0
None
Survivin AS
Control
Migrated cells/field
0
1
10
100
VEGF (ng/ml)

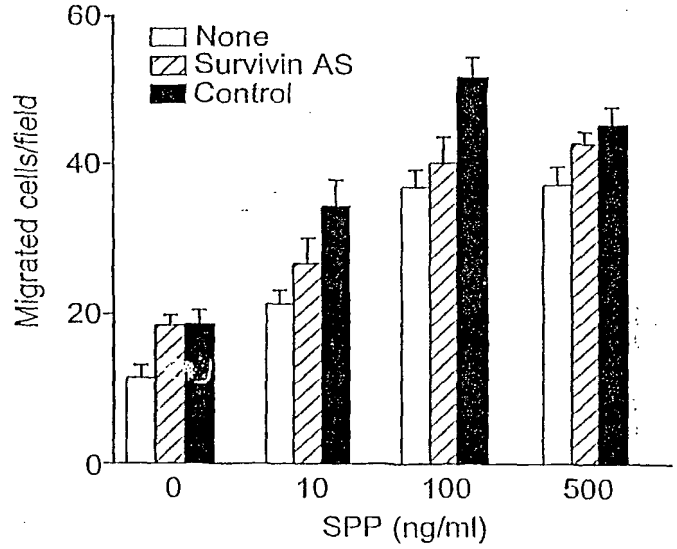

60
40
20
0
None
Survivin AS
Control
Migrated cells/field
0
10
100
500
SPP (ng/ml)

Figure 14

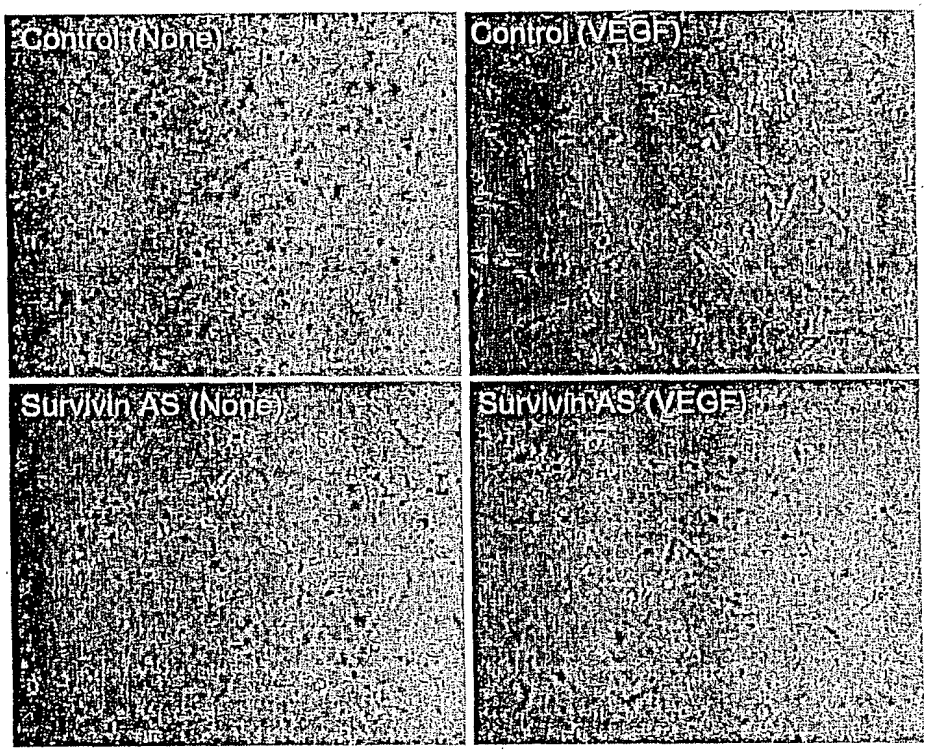

Control (None)
Control (VEGF)
Survivin AS (None)
Survivin AS (VEGF)

Figure 15

SEQUENCE LISTING

<110> Altieri, Dario C.
Sessa, William C.
Yale University

<120> Survivin Promotion of Angiogenesis

<130> 44574-5056-WO

<140>
<141>

<150> US 60/172,991
<151> 1999-12-21

<160> 2

<170> PatentIn Ver. 2.1

<210> 1
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Survivin antisense oligonucleotide

<400> 1
tgtgctattc tgtgaatt 18

<210> 2
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Control (scrambled) oligonucleotide

<400> 2
taagctgttc tatgtgtt 18